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Migration Stimulating Factor: New Marker for Tumour Progression?

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MIGRATION STIMULATING FACTOR:

NEW MARKER FOR TUMOUR

PROGRESSION?

Thesis submitted by

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Master's degree in MEDICAL BIOTECHNOLOGY

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(International Ph.D. Programme in Immunology and Immunopathology)

For the Degree of

Doctor of Philosophy

Under the supervision of

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2015

DATE OF SUBMISSION: 28 APRIL 2015

DATE OF AWARD: 19 JUNE 2015

Abstract

The aims of my work were: a) to generate reagents (recombinant protein and antibodies) useful to identify MSF as a possible marker for TAMs in human tumours; b) to characterise the biological properties of MSF. MSF is a soluble oncofoetal isoform of human fibronectin 1, identical to the N-terminus portion of fibronectin and characterized by the presence of a unique 10 amino acids long sequence in the C-terminus end. MSF is expressed by epithelial and stromal cells during foetal development and in cancer patients, but not by adult normal cells. Our group found a selective expression of MSF by M2-polarized human macrophages and M2-like tumor-associated macrophages (TAMs). TAMs are major inflammatory cells infiltrating tumors, they support tumour growth and are associated to poor prognosis.

These findings suggest that MSF could be a potential marker for tumor progression associated to M2-like polarization of TAM and a promising target for anti-cancer therapy. Human MSF has been expressed in CHO cells and purified by affinity chromatography. Recombinant MSF has a motogenic effect on cancer cell lines, human monocytes and murine peritoneal macrophages. Different levels of MSF expression were observed in human cancer cell lines and correlate with its motogenic effect. *In vivo* experiments showed that tumors derived from MSF-expressing cells had an higher growth rate compared to tumors derived from control cells. We also developed a monoclonal antibody (MoAb_E10) recognizing human MSF. A initial characterization on human tumour tissues (lung, breast, colon) showed MSF expression in tumor cells as well as stromal cells. In particular MSF expression is associated to M2-like TAM. Given that TAMs are promising target for anticancer therapy, the possibility to identify M2 polarized cells would be of considerable interest and further studies will be necessary to define the importance of MSF in the context of human tumours.

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LIST OF ABBREVIATIONS

Ab	Antibody
BSA	Bovine Serum Albumin
CCL-	CC-chemokine
CCR-	CC-chemokine receptor
CLR	C-Type Lectin Receptor
CTL	Cytotoxic T Lymphocytes
CXCL-	CXC-chemokine
CXCR-	CXC-chemokine receptor
ECM	Extra Cellular Matrix
ED-A	Extra-Domain A Fibronectin
ED-B	Extra-Domain B Fibronectin
EGF	Epidermial Growth Factor
ELISA	Enzyme-Linked Immunosorbent Assay
FGF	Fibroblast Growth Factor
FCS	Fetal Calf Serum
FN	Fibronectin
Gel-BD	Gelatin-Binding Domain of Fibronectin
HNSCC	Head and Neck Squamous Cell Carcinoma
IFN-γ	Interferon- γ
IgG	Immunoglobulin
IL-	Interleukin
kDa	kiloDalton
LPS	Lypopolysacharide
M1	Calssically Activated Macrophages
M2	Alternatively Activated Macrophages
M-CSF	Macrophage- Colony Stimulating Factor
MDSC	Myeloid-Derived suppressor Cell
MHC	Mayor Hystocompatibility Complex
MMP	Matrix Metalloproeinase
MSF	Migration stimulating Factor
NF-κB	Nuclear Factor kappa B
NK	Natural Killer
NKT	Natural Killer T
NLR	Nod-Like Receptor
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PMN	Polymorphonuclear Leukocytes
PRR	Pattern Recogniiton Receptor
RAG	Recombinant-Activation Gene Knock-Out
RLR	RIG-I-Like Receptor
SD	Standard Deviation
SE	Standard Error
STAT3	Signal Transducer and Activator of Transcription 3
SPARC	Secreated Protein Acidic and Rich in cystein
TAN	Tumour-Associate Neutrofils
TADC	Tumour-Associated Dendritic Cells
TAM	Tumour-Associated Macrophages
TC-Mφ	Tumour- Contioned Macrophages
TEM	Angiopoietin-2 receptor Tie2
TGF	Transforming Growth Factor
TIL	Tumour Infiltrating Lymphocyte

TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor
TME	Tumour Microenvironment
VEGF	Vascular Endothelial Growth Factor

INDEX

1 INTRODUCTION

1.1 The Immune System

The mammalian immune system is composed of two branches: innate and adaptive immunity, which cooperate to protect the host against microbial infections, injury, sterile wound healing, and to maintain homeostatic balance of tissues. Innate immunity is evolutionarily the oldest mechanism of defence against microbes and is present in many species, from primitive multicellular organisms to vertebrates (Hoffmann & Akira, 2013). However, during evolution, the development of adaptive immunity allowed the immune system of vertebrates to recognize and neutralize more efficiently and rapidly invading pathogens.

In adaptive immunity, pathogen-specific receptors are "acquired" during the lifetime of the organism, whereas in innate immunity receptors recognizing pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, peptidoglycan, lipotheichoic acids and cell-wall lipoproteins, are already encoded in the germline. Through these receptors the adaptive immune response mediates effective and specific attacks against microorganisms, in cooperation with the innate immune system (Hoffmann & Akira, 2013). In the adaptive immune system, the generation of highly diversified receptors is the result of somatic recombination of germline-encoded segments, and provides high recognition specificity for foreign antigens, immunological memory of infections and development of pathogen-specific adaptor proteins; this mechanisms is mediated by Histocompatibility Complex (MHC) molecules (Medzhitov, 2007). However, as the adaptive immune system is able to mediate the anti-microbial response, it may also be responsible for allergy, autoimmunity and the graft versus host disease (Janeway & Medzhitov, 2002).

The innate immune system, also known as the non-specific immune system, represents a first-line host defence mechanism. It is composed of a cellular and a humoral arm. The

cellular arm comprises primarily leukocytes, such as neutrophils and macrophages that mediate the early phase of the innate immune response to the infectious agents crossing the structural barriers of the organism (Medzhitov, 2007). On the other hand, the humoral arm includes soluble molecules, such as complement components, lectins, pentraxins and defensins, produced and released by innate immune cells (Bottazzi et al, 2010). When a given pathogen invades the host, it is initially recognized by cells of the innate immune system. In the case the innate immune system is not successful in removing the pathogen, it can activate the adaptive immune system to efficiently eradicate the infection. The recognition of pathogens can be also performed by non-immune cells such as epithelial cells, fibroblasts, and endothelial cells.

Vertebrates sense microbial invasion by utilizing various pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), Nod-like receptors (nucleotide-binding oligomerization domain receptors - NLRs), RIG-I-like receptors (intracellular pattern recognition receptor - RLRs), and C-type lectin receptors (CLRs) (Hoffmann & Akira, 2013). These PRRs are located on the cell surface and/or in the cytoplasm. They activate a series of events leading to the production by the innate immune cells of several cytokines and chemokines (IL-1; IL-6; IL-10; TNF; CCL2), and to the recruitment and accumulation of leukocytes and immune cells involved in the inflammatory response (such as neutrophils and macrophages). Basically, the inflammatory response is initially promoted by dendritic cells expressing microbe-derived peptides associated with the major histocompatibility complex (MHC) on the cell surface (Hoffmann & Akira, 2013). Dendritic cells migrate from the infected tissues to the regional lymph nodes, where they present the antigen to specific T lymphocytes. This results finally in the activation of adaptive immunity (Hoffmann & Akira, 2013). Moreover, the innate immune system has evolved to be activated by microbes, therefore autoimmunity events that might be caused by an improper activation of the adaptive system are unlikely to be the result of the innate immunity activation (Medzhitov, 2007). It is remarkable that these innate responses not

only play a key role as first line of defence against pathogens, in the homeostasis and maintenance of tissue integrity, but are also involved in the activation and regulation of adaptive responses.

1.2 The Immune System and Cancer

It is now accepted that the immune system plays at least three distinct roles in preventing cancer: (i) it protects the host against viral infections and hence suppresses virus-induced tumours; (ii) it prevents the establishment of an inflammatory environment that facilitates tumorigenesis by promoting a prompt resolution of sterile and pathogen-induced inflammation; and (iii) it eliminates tumour cells in certain tissues, recognized thanks to the expression of ligands for activating receptors on innate immune cells and tumour antigens recognized by the adaptive immune system (Schreiber et al, 2011).

1.2.1   Cancer immunosurveillance

Accordingly to the cancer immunosurveillance hypothesis, cells of the immune system could be able to recognize and destroy continuously arising transformed cells. This hypothesis was tested over the years to determine whether tumour development is influenced by an impaired immune system (see for review (Dunn et al, 2002; Schreiber et al, 2011). Of particular note were experiments showing that the cancer susceptibility (spontaneous or carcinogen-induced tumour) of immunocompetent mice was similar to that of nude mice that had major but not total immunodeficiency (Stutman, 1975). Following this evidence, the cancer immunosorveillace hypothesis was abandoned and have been brought forward other hypotheses according to which tumour cells did not possess "danger signal" needed to activate immune system (Matzinger, 1994), or immune system would ignore or be tolerant to a progression of tumour because tumour cells are very similar to normal cells (Pardoll, 2003). On the other hand, others demonstrated that a persistent activation of a pro-inflammatory arm of innate immunity could facilate tumour progression promoting tumour proliferation, compromising the protective function of the

innate system (Balkwill & Mantovani, 2001; Karin et al, 2002). Despite the controversial observations, due mainly to the limited models utilized at that time, by the 1990s improved mouse model of immunodeficiency allowed to reassess the role of immunity on cancer development.

A number of discoveries led to a renewed interest in the idea of cancer immunosurveillance. First of all, endogenously produced interferon γ (IFN-γ) was demonstrated to protect the host against the development of tumours, both chemically induced and spontaneous (Dighe et al, 1994; Kaplan et al, 1998). Secondly, mice lacking perforin (*perforin-/-*), a cytolytic protein found in the granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, were more prone to develop tumours (van den Broek et al, 1996), demonstrating, together with the first observation, that the immune system is involved in the control of tumour formation. Moreover, using Recombinant-Activation Gene knock-out (RAG-/-) mice, which completely lack natural killer T (NKT), T and B cells, it was demonstrated that lymphocytes were not only necessary to protect the host against chemically induced sarcomas but also against spontaneous tumours (Shankaran et al, 2001; Shinkai et al, 1992). The tumour suppressor mechanisms dependent on IFN-γ, perforin and lymphocytes are heavily overlapped indicating that both the innate and the adaptive arm of the immune system are involved (Dunn et al, 2002; Mittal et al, 2014; Schreiber et al, 2011).

On the other side it has been shown that the immune system can lead to the immunoselection of tumour cells that are more capable of surviving in an immunocompetent host (Dunn et al, 2002; Mittal et al, 2014; Schreiber et al, 2011). Based on this, the immune system exerts an apparent paradox that consists of two opposite effects on developing tumours: being on one side protective for the host and on the other promoting tumour growth. In particular, cancer immunosurveillance is thought to act at the earliest stages of cellular transformation, whereas, the tumour editing effect can become

overwhelming, at late stages. This process, in all its features, could no longer be named "immunosurveillance", and the term "immunoediting" was proposed (Dunn et al, 2002). Cancer immunoediting is the result of three processes: elimination, equilibrium and escape, a concept known as the "three Es" (Figure 1).

The elimination process corresponds to the original concept of cancer immunosurveillance and during this phase the immune system recognizes and tries to destroy the transformed cells (Matzinger, 1994). If the elimination of all these cells is successful, there is no progression to the next step and the tumour is eradicated without any clinical manifestation (Dunn et al, 2002; Mittal et al, 2014; Schreiber et al, 2011; Vesely & Schreiber, 2013). In particular, cellular transformation leads to the induction of an inflammatory response with recruitment of cells of the innate immune system: NK cells, NKT cells and γδT cells recognize structures on the transformed cells and initiate production of IFN-γ, which in turn can exert an apoptotic and antiproliferative effect on tumour cells (Dunn et al, 2002; Matzinger, 1994; Schreiber et al, 2011). Moreover, IFNγ also induces the secretion in the microenvironment of pro-inflammatory anti-angiogenic chemokines, such as CXCL9, CXCL10 and CXCL11, by tumour cells and other host cells. Chemokines sustain the recruitment of NK cells and monocytes, which differentiate into macrophages. Recruited cells can thus contribute to the elimination of tumour cells. In parallel, local dendritic cells engulf tumour cell debris and migrate to draining lymph nodes, where they activate CD4+ and CD8+ T cells. Activated T cells migrate to the tumour site, where antigen-bearing tumour cells can be recognized and destroyed by cytolytic lymphocytes.
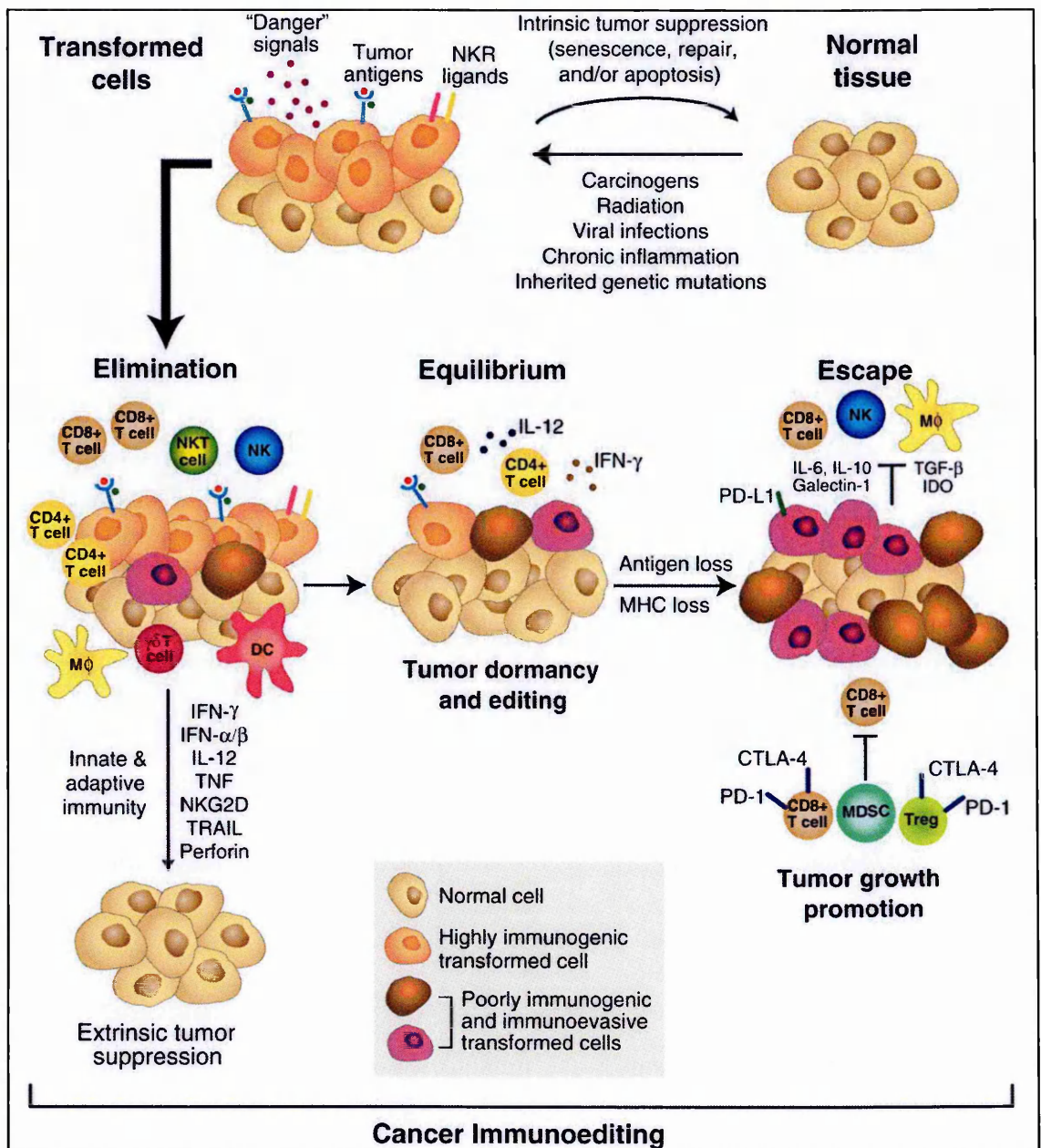
Figure 1: Cancer immunoediting concept. Cancer immonoediting consists of three phases: elimination, equilibrium, and escape. In the elimination phase, the immune system recognizes and destroys tumors before they become clinically apparent. If a rare cancer cell variant is not destroyed in the elimination phase, it may then enter the equilibrium phase. The constant immune selection pressure, placed on tumor cells genetically unstable can promote the appearance of rare cancer cell variants no longer recognized by the immune system. Such cells may thus enter the escape phase. During the escape process, the uncontrolled expansion of the surviving tumor variants occurs resulting in the clinical manifestation of the malignant desease. Figure taken from Schreiber *et al.* 2011.

The equilibrium phase is a dynamic process generated by the activity of the immune system itself on the tumour mass (Schreiber et al, 2011). Equilibrium can become the longest phase of cancer immunoediting, eventually extending throughout the life of the host. During the equilibrium phase, growth of residual tumour cells is kept under control by the immune system, maintaining transformed cells in a "dormant state" for a long time, until they accumulate a combination of alterations sufficient to allow them to evade immune recognition and resume their growth as either recurrent primary tumours or distant metastases (Aguirre-Ghiso, 2007). Evidence for the requirement of a functional immune system to sustain the equilibrium phase came from primary tumorigenesis experiments showing that administration of a low-dose of carcinogen [3methylcholanthrene (3-MCA)] to immunocompetent mice did not result in the development of tumours, despite cancer cells being maintained for an extended period of time (Koebel et al, 2007). However, ablation of the immune system in these mice by administration of monoclonal antibodies (mAbs) that deplete T cells and IFN-γ, resulted in a rapid tumour outgrowth. Therefore, equilibrium is the result of growth inhibition and cytotoxic activity mediated by the immune system on the residual tumour cells. Nevertheless, the constant activity and suppression of tumour expansion by the immune system, represents a selective pressure that pushes tumour cells to acquire immuneevasive mutations, leading to what is defined evasion or "escape" (Mittal et al, 2014).

In the escape process, the uncontrolled expansion of the surviving tumour variants occurs and this results in the clinical manifestation of the malignant disease (Mittal et al, 2014; Schreiber et al, 2011). Tumour cell escape can occur through many different mechanisms (Dunn et al, 2002; Mittal et al, 2014; Vesely et al, 2011). Alterations leading to reduced immune recognition or increased resistance to the cytotoxic effects of the immune system (for example, through induction of anti-apoptotic mechanisms or expression of anti-apoptotic effector molecules such as BCL-2) promote tumour outgrowth (Khong & Restifo, 2002; Smyth et al, 2006). Moreover, tumour cells can develop at least

three different ways to evade immune recognitions: 1) silencing the expression of tumour associated antigens, 2) preventing the expression of MHC class I proteins that present these antigens to tumour-specific T cells, or 3) hampering the antigen processing machinery necessary for the association of tumour antigens with MHC class I molecule. All of these alterations might be, at least in part, the result of on-going genomic instability, which is a common feature of transformed cells. The final outcome of the selection of poorly immunogenic tumour cell variants, no longer sensed by the immune system, unleashed to grow indefinitely.

1.2.2 Cancer-related inflammation

Inflammation is a complex immune editing process that normally protects the organism from invading pathogens and preserves homeostasis in the presence of tissue damage (Medzhitov, 2008). It is now generally accepted that inflammation has a role in tumorigenesis (Balkwill & Mantovani, 2012). Chronic inflammation has been shown to contribute to tumorigenesis at different stages: i) tumour initiation, by inducing genotoxic stress; ii) tumour maintenance, by promoting cell growth and proliferation; iii) tumour progression, by enhancing angiogenesis and tissue invasion (Grivennikov et al, 2010). Therefore, it has been proposed that inflammation and tumour immunity are mutually exclusive (Balkwill & Mantovani, 2012). Epidemiological studies have shown that chronic inflammation can increase the risk of cancer leading to the definition of cancer-related inflammation (Balkwill & Mantovani, 2012; Mantovani et al, 2008b). However, other evidence is available supporting the idea that tumour-promoting inflammation and protective tumour immunity might be reciprocally correlated (Bui & Schreiber, 2007). In fact, if on one side the immune system is able to "shape" cancer cells during immunoediting, the tumour mass can have a strong influence on the immune system itself. Thus, inflammation and tumour immunity might be considered a dynamically interconnected process in which tumour cells take over through cancer immunoediting.

This hypothesis is supported by various data: development of chemically induced tumours (such as upon treatment with 3-MCA) is dependent on the presence of pro-inflammatory cytokines/signalling molecules [e.g. interleukin-1β (IL-1β), IL-23 or MyD88]; once the tumour is formed, growth of transformed cells can be controlled by other cytokines (such as IFNγ, IFNα/β, IL-12) or T cells. Moreover, components of the immune system showing pro-oncogenic activity can also induce tumour immunity during the development of cancer: MyD88 and IL-1β promote carcinogen induced-tumorigenesis in mouse models (Krelin et al, 2007; Swann et al, 2008). In addition, at later stages of tumour development the same proteins exert a protective response against tumours, promoting the recognition of transformed cells (Balkwill & Mantovani, 2012; Ghiringhelli et al, 2009). Finally, studies both in mouse models and *Drosophila* showed that tumour-necrosis factor (TNF) can have both tumour-promoting and anti-tumour activities (Cordero et al, 2010), and that IFNγ is required both for Ultraviolet B-induced tumour formation and for immune rejection of the same tumours (Zaidi et al, 2011). Therefore, inflammation provides an important contribution to tumour escape; i.e. when inflammatory cells are recruited to the site of a growing tumour, they are activated by the paracrine production of cancer-related molecules, such as vascular endothelial growth factor (VEGF), and repress immune responses against the tumour itself (Grivennikov et al, 2010).

Tumour initiation, as well as the genetic and epigenetic aberrations leading to transformation of normal cells, are dramatically favoured by chronic inflammation (Mantovani et al, 2008a). Its pro-tumoral contribution is exerted by establishing a tissue microenvironment favourable for tumour growth, and by preventing an effective anti-tumoral immune response (Trinchieri, 2012). Accordingly, in the tumour microenvironment (TME), inflammatory cells and molecules influence almost every aspect of cancer progression and as shown in Figure 2. Based on this, cancer-related inflammation has been added as the seventh hallmark of cancer (Colotta et al, 2009).
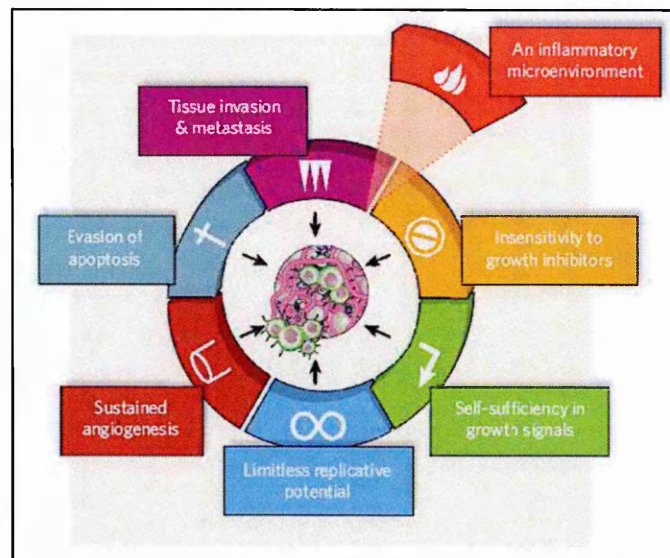
Figure 2. Hallmarks of cancer The hallmarks of cancer proposed by Hanahan and Weinberg (2000) include: unlimited replicative potential, ability to develop blood vessels (angiogenesis), evasion of programmed cell death (apoptosis), self-sufficiency in growth signals, insensitivity to inhibitors of growth, and tissue invasion and metastasis. The inflammatory microenvironment has been recently proposed as the seventh hallmark of cancer. Taken from Colotta *et al*, 2009.

1.2.3   Intrinsic and extrinsic pathways in inflammation and cancer

Inflammation and cancer are interlinked in two main ways: intrinsic and extrinsic pathways (Figure 3). Inflammation associated with cancer initiation is defined as *intrinsic* when the mechanisms that are involved in cell transformation, typically oncogene over-expression or mutations, are also responsible for the activation of a pro-inflammatory programme (Karin, 2006). On the other hand, *extrinsic* inflammation is the result of tissue response to malignant cells, and is most predominantly mediated by infiltrated inflammatory cells. (Mantovani et al, 2008b; Trinchieri, 2012).

Causes of the intrinsic pathway of inflammation are re-arrangements of genes implicated in the tyrosine-kinase pathways, mutations in the RAS family proteins and in the oncogene MYC (encodes a transcription factor), which are not only necessary and sufficient for cancer development, but also directly promote the generation of inflammatory conditions (Guerra et al, 2007; Soucek et al, 2007). Uncontrolled proliferation and survival of cells, epithelial-to-mesenchymal transition, angiogenesis,

tumor cell migration and metastasis, altered response to hormones and chemotherapeutic agents, are expected to be observed as downstream effects of oncogenic variations (Mantovani et al, 2008b). As shown in Figure 3, the early genetic events are able to induce the expression of cytokines, chemokines, prostaglandins and thus to instruct the remodelling of the microenvironment, which will in turn promote tumour progression, invasion and metastasis (Mantovani et al, 2009).
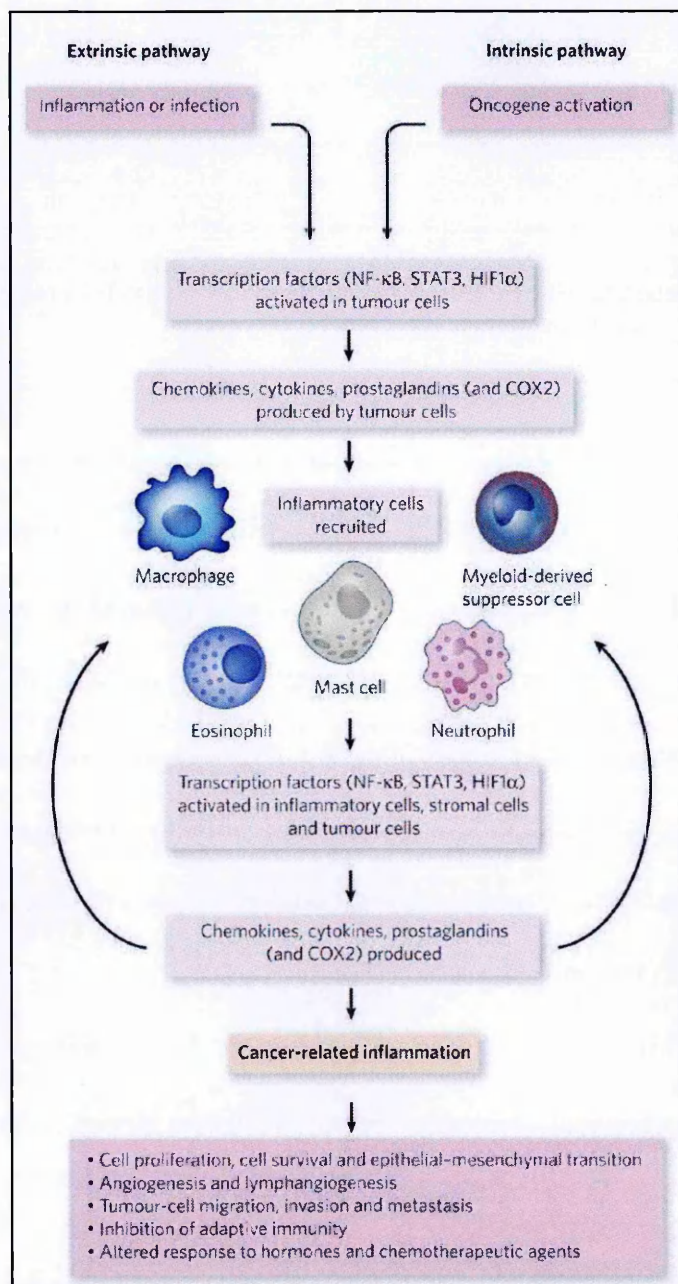
Figure 3. Pathways that connect inflammation and cancer. Inflammation and cancer are connected by two pathways: an intrinsic pathway, driven by oncogenic mutations that cause both inflammation and neoplasia, and an extrinsic pathway, driven by inflammatory conditions that favors the tumor development. Figure taken from Mantovani *et al*, 2008

The interplay between the intrinsic and extrinsic inflammatory pathways can be exemplified in the case of pancreatic carcinoma, in which both an extrinsic inflammatory condition (pancreatitis) and the mutation in the gene encoding K-RAS (intrinsic pathway) are necessary to drive carcinogenesis (Borrello et al, 2005; Guerra et al, 2007). The intrinsic pathway is able to promote inflammation through the activation of endogenous key transcription factors such as Nuclear Factor kappa-light-chain enhancer activated B cells (NF-κB) and Signal Transducer and Activator of Transcription 3 (STAT3) (DiDonato et al, 2012; Yu et al, 2007). NF-κB, in particular, has emerged as an important tumour promoter. This transcription factor is activated upon engagement of Toll-like receptors by microorganisms or damaged tissues (TLR-Myd88 signalling pathway) (Mantovani et al, 2008a). It regulates the production of several inflammatory cytokines, chemokines, adhesion molecules and angiogenic factors. In addition it activates the prostaglandin synthesis pathway and promotes cell survival and growth, inhibiting apoptosis and favouring the response to hypoxia (DiDonato et al, 2012). These inflammatory conditions promote leukocyte infiltration, which occurs in most tumours. Tumour infiltrating leukocytes are mainly myeloid cells and their subpopulations [tumour associated macrophages (TAMs), monocytes expressing the angiopoietitn-2 receptor Tie2 (TEM), myeloid derived suppressor cells (MDSC), tumour associated neutrophils (TAN) and tumour-associated dendritic cells (TADC)], mast cells, T cells, NK cells, dendritic cells (Balkwill, 2004; Brennecke et al, 2014). Importantly, it has to be considered that many cells residing in the inflamed tissue where the tumour originated, including the surrounding non-transformed epithelial and stromal cells, are affected by the environmental stimuli locally generated (Brennecke et al, 2014). In this environment, genetic instability is fostered leading to the selection of mutation(s) responsible for the expansion of a single tumour clone. At the same time the normal counterpart will remain

quiescent or will die because of senescence, or destruction by innate and adaptive immune cells, but also an excessive DNA damage could cause cell death.

1.3 The tumour microenvironment

Cancer is a complex "rogue" organ in which malignant cells and host cells coexist, and contribute to the formation of the tumour microenvironment (TME). Host cells in the tumour microenvironment include fibroblasts, endothelial and immune-competent cells (Hanahan & Coussens, 2012). All together tumour and host cells orchestrate molecular and cellular events taking place in surrounding tissues (Figure 4).
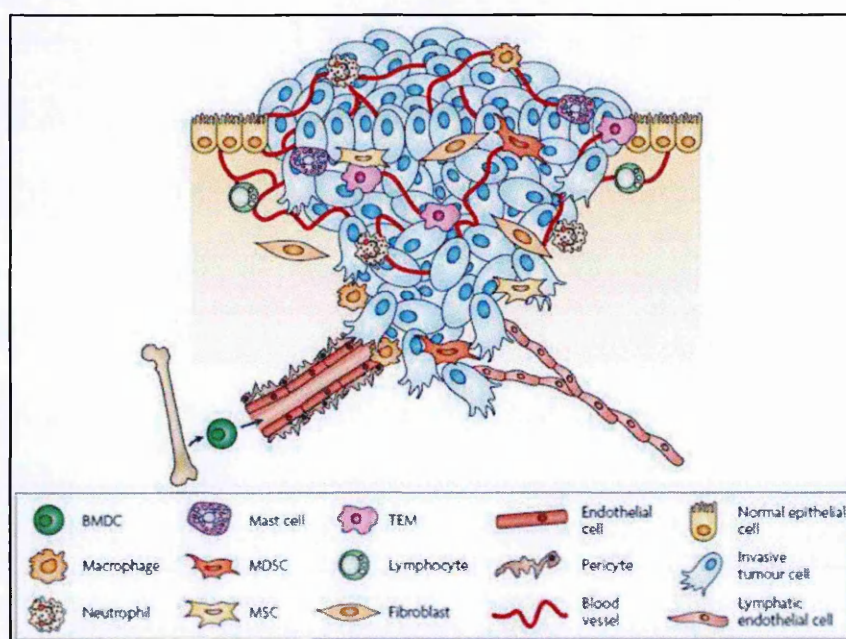
Figure 4. The primary tumour microenvironment. TME is characterized by a huge infiltrate of immune cells (macrophages, neutrophils, lymphocytes), by new-born blood vessels and deregulated ECM proteins Figure taken from Pollard *et al.*, 2009.

Besides host cells, a wide array of biologically active molecules are available in the tumour microenvironment in soluble form or associated to component of the extracellular matrix (ECM) (Bornstein & Sage, 2002). ECM is an essential component of TME and it is composed by fibrous proteins (e.g. collagen, elastin, cheratin) embedded in a gel of glycosaminoglycans, proteoglycans and glycoproteins. It not only constitutes the structural scaffold for cellular elements of the stroma, but also exerts a direct influence on cell

growth, differentiation and migration (Schultz et al, 2011). Several ECM molecules, defined as "matricellular proteins" by Bornstein and Sage, are able to interact with cell-surface receptors and with other microenvironment components. These include cytokines, chemokines, growth factors and proteases, that altogether can affect various cell functions. Interestingly, the idea that tumour stroma may have an impact on the biological behaviour of neoplasms is as old as the first histological observation of a neoplasia (Dhom, 1994).

The extensive remodelling of the tumour ECM compartment is mainly carried out by macrophages, a key cell of the TME (Brennecke et al, 2014). They act through two main processes: (i) the degradation of pre-existing ECM molecules by a number of hydrolytic enzymes (e.g. proteases) produced, activated and /or induced by neoplastic cells and (ii) the *de novo* synthesis of ECM components, which, in many cases, are not present or are present at low concentrations in normal tissue. Transforming growth factor β (TGF-β) is a potent inducer of ECM proteins such as collagens, tenascin, thrombospondin, osteopontin, elastin, the secreted protein acidic and rich in cysteine (SPARC) and fibronectin (Roberts et al, 1992). Such modified tumour ECM could generate a more suitable, permissive and /or instructive environment for tumour growth and progression (Lu et al, 2012).

The evolution, structure and activities of the cells in the TME have many parallels with the processes of wound healing and inflammation (Mantovani et al, 2008b). However cells such as macrophages are also found in cancers that have no known association with chronic inflammatory conditions (Grivennikov et al, 2010; Hanahan & Coussens, 2012; Mantovani et al, 2008b). This because inflammatory and wound-healing processes can be also activated downstream of oncogenic mutations in the malignant cells (Mantovani et al, 2008b).

Inflammation is a consistent feature of the tumour microenvironment, and as described above has been considered the seventh hallmark of cancer (Balkwill & Mantovani, 2012; Colotta et al, 2009; Coussens et al, 2013; DiDonato et al, 2012; Mantovani et al, 2008b). As suggested by recent estimates, 25% of cancers are associated with chronic

inflammation sustained by infections (e.g. hepatitis) or inflammatory conditions of diverse origin (e.g. prostatitis) (Balkwill & Mantovani, 2012). In addition, even tumours not directly connected to inflammation are characterized by the presence of cells and mediators of the inflammatory response (Grivennikov et al, 2010).

1.3.1 Cells of the tumour microenvironment

As mentioned above, host cells, including leukocytes, fibroblast and endothelial cells, infiltrate tumours (Brennecke et al, 2014). Leukocytes, and in particular myeloid cells, are the most consistent cellular infiltrate of solid tumours. Tumour associated myeloid cells (TAMC) mainly support tumour growth and progression, thereby contrasting the T-cell infiltrate, which mainly has anti-tumoral activity (Brennecke et al, 2014).

TAMC all arise from hematopoietic stem cells (HSC) within the bone marrow, and further differentiate into macrophage/granulocytes progenitors. The myeloid populations that infiltrate tumours modulate tumour-mediated immunosuppression, tissue remodelling, tumour progression and metastasis (Mantovani & Sica, 2010). Similarly to tissue macrophages, TAMC demonstrate high plasticity, resulting in the two extreme M1 and M2 phenotypes for polarized macrophages and N1 and N2 phenotypes for polarized neutrophils (see below) (Mantovani, 2009; Mantovani et al, 2005; Qian & Pollard, 2010). Cross-talk between the different cellular components was demonstrated, resulting in tuning of the adaptive immune response, promotion of angiogenesis and tissue remodelling (Mantovani & Sica, 2010).

Results obtained so far clearly indicate that TAMC are major players in the connection between inflammation and cancer. Recent work has led to a better understanding of their biological properties, indicating that myeloid cells infiltrating growing tumours could have a prognostic value, thus representing an attractive target for novel biological therapies of tumours (Fridman et al, 2012).

Although cancer-related inflammation is considered to be tumour-promoting, as

described above, the immune-surveillance of cancer does exist: inflammatory cells can recognize and destroy tumour cells, following the immunoediting theory (Schreiber et al, 2011). The tumour mass is able to shape the immune system and the balance between tumour-promoting (Th2 cells and M2 macrophages) and tumour-destructive (Th1 and M1 macrophages) immune response is perhaps significant for cancer development (Mantovani & Sica, 2010). A key point that needs to be addressed in future studies, is how to trigger a "good" immune response during anti-cancer therapy. Tumour associated macrophages seem to have a crucial role in the generation of a tumour-promoting microenvironment, whereas M1 macrophages are necessary for the elimination of tumour cells and elicit cancer-inhibiting immune response (Mantovani et al, 2008b). Re-education of macrophages could be a good target to reverse the tumour-supporting activity (M2-like) of these cells towards antitumor function (M1- like).

As already mentioned, tumours are highly infiltrated by different types of immune cells that profoundly contribute to the shaping associated of micro-environment (Brennecke et al, 2014). Macrophages, dendritic cells, neutrophils, lymphocytes and myeloid-derived suppressor cells (MDSC), all seem to play crucial activities within the malignant context, as well as fibroblasts, pericytes and sometimes adipocytes.

1.3.1.1   Tumour-associated macrophages (TAMs)

Tumour-Associated Macrophages (TAMs) belong to the early infiltrating leukocyte population within tumours, thus preceding lymphocytes, and are usually the most abundant immune population in the tumour microenvironment, representing up to 50% of the tumour mass (Mantovani et al, 2008b; Qian & Pollard, 2010). TAMs are fundamental actors in tumour growth, where their activities are usually pro-tumorigenic.

Monocytes are known to originate in the bone marrow under the influence of interleukin-1α (IL-1α), IL-6, IL-13 (Geissmann et al, 2010) and macrophage-colony stimulating factor (M-CSF or CSF-1), which stimulates their differentiation, as well as their proliferation and viability *in vitro*, from a common myeloid progenitor. Various

molecules produced either by neoplastic and by stromal cells are involved in the recruitment of monocytes from the blood to the tumour site. Monocyte the differentiation is also influenced by their localization within the tumour mass. For instance, a gradient of IL-10 can switch monocyte differentiation towards macrophages rather than dendritic cells (Allavena et al, 1998). Thus, as observed in breast cancer and papillary carcinoma of the thyroid, TAMs are present throughout the tissue whereas DC are present only in the periphery (Scarpino et al, 2000).

Monocytes express the of M-CSF receptor (MCSF-R, CD115) which is a useful marker to define these cells (Chitu & Stanley, 2006). Monocytes also express a wide range of scavenger receptors and upon stimulation they release reactive oxygen species (ROS), complement factors, prostaglandins, nitric oxide (NO) and cytokines (e.g. TNF, IL-1β, CXCL8, IL-6, and IL-10), VEGF and proteolytic enzymes (Auffray et al, 2009). Moreover, they express chemokine receptors (e.g. CXCR1) and adhesion receptors mediating migration from blood to tissues during inflammatory/infection conditions.

Monocytes are effectors of the immune response to microbial infections, are implicated in many inflammatory disease and can be recruited into tumour sites, where they are involved in the regulation of tumour development (Peranzoni et al, 2010). Once released into the peripheral blood, monocytes circulate for several days before homing to tissues and replenishing macrophage populations. Macrophages differentiate into subpopulations of tissue macrophages and closely related myeloid dendritic cells (Steinman & Idoyaga, 2010), the latter specialized to present antigens to naïve T helper lymphocytes, contributing to host defence, tissue remodelling and repair (Auffray et al, 2009).

In the steady state, in the absence of overt inflammation, resident macrophages populate organs constitutively and mediate trophic and homeostatic roles, e.g. removing apoptotic cells and serving as sentinels of injury and infection (Gordon & Martinez, 2010). Once an early inflammatory response, such as acute infection, has occurred, monocytes rapidly invade injured tissues and differentiate to macrophages. Tissue macrophages are terminally

differentiated cells that show a distinct rate of proliferation depending on the stimuli and the tissue microenvironment. They are biosynthetically active and can recognize foreign, normal or abnormal cells, acting as very efficient scavenging cells.

The chemokine CCL2 (also known as monocyte chemotactic protein 1; MCP-1), described in 1983 as a tumour-derived chemotactic factor, is the main player involved in macrophage accumulation within tumours (Bottazzi et al, 1983; Negus et al, 1995). Several studies have correlated CCL2 levels with TAM abundance in many experimental and human tumours such as ovarian, breast and pancreatic cancer (Balkwill, 2004; Bottazzi et al, 1983). TAM themselves produce CCL2, suggesting the action of an amplification loop (Solinas et al, 2009). Other chemokines involved in monocytes recruitment are CCL5, CCL7, CXCL1 and CXCL12, as well as such as urokinase plasminogen activator (uPA), TGF-β, basic fibroblast growth factor (bFGF), VEGF and platelet derived growth factor (PDGF), as well as antimicrobial peptide (e.g. human beta-defensin-3) and the growth factor M-CSF (Allavena et al, 2008b; Balkwill, 2004). Moreover, monocytes can also be attracted by fibronectin 1 (FN1), fibrinogen and other factors produced during the cleavage of ECM proteins induced by macrophages and/or tumour cell-derived proteases (Allavena et al, 2008a; DeNardo et al, 2008).

Early studies demonstrated that appropriately stimulated macrophages are able to kill tumour cells *in vitro* (Erreni et al, 2011). However TAM, conditioned by the tumour microenvironment, lose their cytotoxic capability, and rather exert several pro-tumoral functions, mediating cancer-related inflammation, angiogenesis, immune-suppression, tissue remodelling and metastasis (see below) (Mantovani et al, 2008b; Pollard, 2004).

1.3.1.2 Dendritic cells

Dendritic cells are heterogeneous professional antigen-presenting cells of bone-marrow origin that are critical for the initiation of primary T-cell response (Steinman, 1991). There is evidence that DCs play a key role in the induction of tumour-specific immune responses, especially via cross-priming which allows the transfer of antigens from tumour cells to

DCs, their presentation through MHC-class I antigens and the generation of $CD8^+$ cytolytic T cells.

In the TME, DCs are defective, that is, they cannot adequately stimulate an immune response to tumour-associated antigens (Gabrilovich, 2004). In patients with cancer, it has been shown that rapidly growing tumours contain small numbers of DCs and that those cells usually have the phenotype of immature DCs (Brennecke et al, 2014). These DCs express no or low levels of the co-stimulatory molecules CD80 and CD86; e.g., DCs derived from melanoma metastases do not express CD86. Tumours produce immunosuppressive factors that block maturation and impair differentiation of DCs. It has also been proposed that the high numbers of immature DCs found in tumours is due to increased migration. In fact, tumour-derived cytokines and chemoattractant factors controlling DCs infiltration (MIP-3α/CCL20) were shown to modulate their recruitment. Other data were collected from patients with breast, melanoma, renal cell and prostate carcinoma, indicating that various malignancies could actively recruit immature DCs to the tumour site and impede their differentiation (Bell et al, 1999).

An hypoxic and inflammatory microenvironment has been found to impair DC functions. Several reports have now confirmed that, by releasing IL-10, IL-6, M-CSF, VEGF and prostanoids, tumours are able to prevent DCs differentiation, maturation and function *in vitro* and *in* vivo (Brennecke et al, 2014).

1.3.1.3   Neutrophils

Neutrophils are short-lived white blood cells derived from bone marrow myeloid precursors and attention has long been focused on their short-term antimicrobial and tissue-damaging function (Cassatella, 2006). Cytokines, including those involved in tumour progression such as IL-1β, dramatically prolong their survival and reprogram their function. Moreover, polymorphonuclear leukocytes (PMN) are themselves a conspicuous source of inflammatory cytokines (Mantovani et al, 2011).

Elevated levels of tumour-associated neutrophils (TANs) were observed in biopsies from patients with colon adenocarcinoma compared with the surrounding non-tumoral tissue (Tazzyman et al, 2009). In these biopsies, neutrophils were found throughout the tumour but their numbers increased further in invasive and ulcerated areas. Increased numbers of neutrophils have also been observed in patients with myxofibrosarcoma, gastric carcinoma and melanoma (Mantovani et al, 2011).

Mirroring the M1 and M2 classification proposed for macrophages, neutrophils have been recently divided into N1 and N2 cells (Mantovani et al, 2011). N1 neutrophils exert anti-tumorigenic activities that include the expression of more immunoactivating cytokines and chemokines, low levels of Arginase 1 (Arg1), will the capability of killing tumour cells *in vitro*. On the other hands, the N2 polarization driven by TGF-β has been associated with a protumorigenic phenotype (Fridlender et al, 2009).

In untreated malignancies, TAN have been reported to support neoplastic growth by producing angiogenic factors and matrix-degrading enzymes (Galdiero et al, 2013), by supporting the acquisition of a metastatic phenotype and by suppressing the antitumor immune response (Schmielau & Finn, 2001). These observations are consistent with the hypothesis that most TANs appear to have an N2 phenotype and thus contribute to tumour growth and immunosuppression. Depleting these protumorigenic N2 neutrophils would thus be expected to inhibit or reduce tumour growth, as shown by several studies (Fridlender et al, 2009; Nozawa et al, 2006).

In particular, Fridlender has reported that in lung cancer and mesothelioma models, CD8^{+} T cell activation results in increased recruitment of N1 neutrophils with anti-tumour activity (Fridlender et al, 2009). Conversely, depletion of N2 polarized neutrophils results in retardation of tumour growth.

Preliminary data has suggested that at least part of the source of neutrophil-attracting chemokines is from tumour-associated macrophages, indicating a strong interaction between these two tumour infiltrating immune populations (Mantovani, 2009).

1.3.1.4 *Lymphocytes*

More than 100 years ago, malignant tumours were first noted to contain variable numbers of lymphocytes (Balkwill & Mantovani, 2012), which are known as tumour infiltrating lymphocytes (TILs). Initially, these TILs were thought to reflect the origin of cancer at site of chronic inflammation (Balkwill & Mantovani, 2001) and later it was debated whether TILs provided a favourable environment for cancer growth or rather reflect the host's attempt to eliminate cancer (Rollins, 2006). A high percentage of tumour-infiltrating lymphocytes is usually associated with better prognosis (e.g. for melanoma, colorectal and ovarian cancer) (Clemente et al, 1996; Curiel et al, 2004; Pages et al, 2005) even though in gastric cancer a high number of TIL seems to correlate with tumour progression (Xie et al, 2008) and in head and neck squamous cell carcinomas (HNSCCs) the role of TILs is still controversial (Uppaluri et al, 2008).

Distinct T cell populations characterize the tumour microenvironment at the invasive tumour margin and in draining lymphoid organs. Among these, CD8+ T cells supported by CD4+ T helper 1 (Th1 cells), which are characterized by the production of IL-2 and IFN-γ, correlate with a good prognosis (Fridman et al, 2012). Contrary to this, other CD4+ T cells, such as Th2 cells producing IL-4, IL-5 and IL-13, which support B cell responses, or Th17 cells, producing IL-17A, IL-17F, IL-21 and IL-22 that favour antimicrobial tissue inflammation, are generally thought to promote tumour growth (Fridman et al, 2012). However they have also been associated with a favourable outcome, as in the case of Th2 cells in breast cancer (Yoon et al, 2010) and Th17 cells in esophageal cancer (Lv et al, 2011).

Over the last few years a number of reports have described elevated numbers of regulatory T (FOXP3+ and CD25+) (Treg) cells inside or in close proximity to tumours, draining lymph nodes and also in peripheral blood of cancer patients (Beyer & Schultze, 2009). There is increasing evidence that Treg cells can migrate into tumours and suppress effective anti-tumour responses in the tumour-microenvironment through the production of

IL-10 and TGF-β and through cell-mediated contact with cytotoxic T-lymphocyte antigen 4 (CTLA4), thus contributing to the growth of human tumours (Campbell & Koch, 2011).

In addition, several mechanisms have been described to explain the conversion of conventional CD4 (+) T cells into Treg cells in the context of human tumours (Beyer & Schultze, 2009). Yet little is known about the molecular and cellular features responsible for the increase and maintenance of elevated levels of Treg cells in cancer (Beyer & Schultze, 2009). Thus, targeting Treg cells provides an attractive therapeutic strategy to potentially influence the suppressed immune response in tumour patients, thereby altering and supporting anti-tumour therapy.

1.3.1.5   Myeloid-Derived Suppressor Cells (MDSC)

Myeloid-derived suppressor cells are a heterogeneous population of immature myeloid cell, that have the ability to suppress T cells functions (Brennecke et al, 2014; Gabrilovich et al, 2012). They derive from myeloid progenitors in bone marrow that have not differentiated into mature granulocytes, macrophages or dendritic cells. MDSC have been isolated from blood, spleen and bone-marrow of tumour-bearing mice. In addition MDSC infiltrate the tumour tissue, where local tumour-associated factors promote their activation. In tumour-bearing mice, two main subset of MDSC were identified: monocytic MDSC (M-MDSC), characterized by $CD11b^+$, $Ly6G^-$, $Ly6C^{high}$, and granulocytic MDSC (G-MDSC), characterized by $CD11b^+$, $Ly6G^{high}$, $Ly6C^-$. M-MDSC and G-MDSC show different functional activities: M-MDSC mediated immune suppression is based on the up-regulation of inducible nitric oxide synthase (iNOS), expression of Arg1 and production of suppressive cytokines, whereas G-MDSC mediated immune suppression is characterized by antigen-specific responses (including ROS release requiring prolonged MDSC and T cell contacts) (Gabrilovich et al, 2012). Moreover M-MDSC possess the ability of differentiating into monocytes (macrophages) and dendritic cells, whereas G-MDSC do not possess this potential (Youn et al, 2008).

Tumour-associated MDSC generally exhibit a M2-like phenotype, while M1 and M2 phenotypes could coexist in some mouse models (Brennecke et al, 2014). Human MDSC have been isolated from blood of patients with glioblastoma, colon cancer, breast cancer, lung or kidney cancer, and are still poorly characterized (Dumitru et al, 2012). Recent studies have proposed that human MDSC have a characteristic $CD34^+$, $CD33^+$, $CD11b^+$, and HLA-DR- profile. Similarly to the murine counterpart, human MDSC are divided into two main subset: monocytic MDSC (M-MDSC), characterized by the expression of CD14, and granulocytic MDSC (G-MDSC), identified by positivity for CD15 (Gabrilovich et al, 2012).

1.4 Macrophages and Cancer

As outlined above, TAMs are the most abundant host cell population infiltrating tumours and are indicators of the strong inflammation present at the tumour sites (Balkwill & Mantovani, 2012; Coussens et al, 2013; DeNardo et al, 2008; DiDonato et al, 2012). Myeloid cells exposed to the tumour microenvironment most frequently promote tumour progression. TAM were shown to promote tumour growth directly through the production of trophic and activating factors by stromal and cancer cells such as epidermal growth factor (EGF), bFGF, VEGF, PDGFβ, and TGF-β (Mantovani et al, 2008b). For example, IL-13 and IL-4 produced by CD4+ T cells infiltrating tumours, led to the production and secretion of EGF by TAM (Aspord et al, 2007). In addition TAMs can promote angiogenesis and resistance to therapies. Plasticity and diversity are hallmarks of myelo-monocytic cells and the regulatory pathways involved in such plasticity can orchestrate the yin-yang interactions between TAM and tumour cells.

1.4.1   Macrophage plasticity

In general, macrophages can be identified by a combination of different markers expressed on their surface, such as CD68, CD14, CD16, CD11b and CD163 (Cook & Hagemann, 2013). Indeed, they are able to acquire different polarization states, in response

to environmental cues, such as microbial products, damaged cells and activated lymphocytes (Biswas & Mantovani, 2010).

Classical (M1) or alternative (M2) polarization represent the two extremes of a *continuum* of functional states, which occur *in vivo* either under physiological conditions, such as ontogenesis and pregnancy, or in pathology, such as allergic and chronic inflammation, tissue repair, infection and cancer (Sica & Mantovani, 2012) (Figure 5).
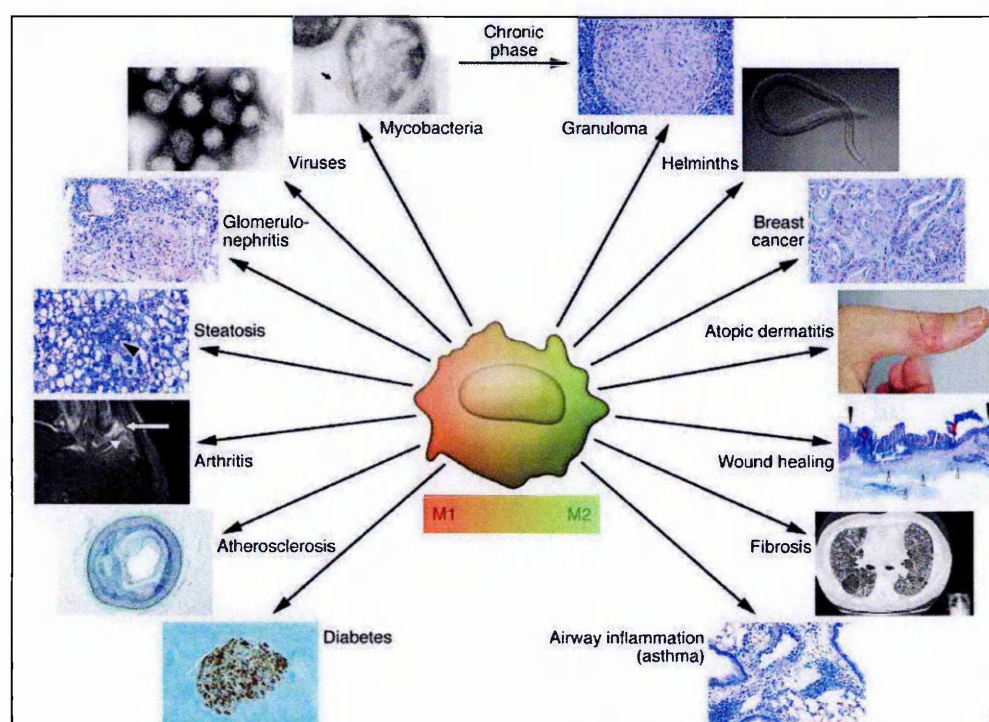
Figure 5. **Macrophage plasticity in human pathologies**. M1 and M2 polarization as the two extremes of a continuum of functional states. Dynamic changes in the functional state are observed in human pathologies. For example, a switch from M1 to M2 macrophage polarization characterizes the transition from early to chronic phases of infections. Taken from Sica and Mantovani,(2012).

Mirroring the Th1/Th2 paradigm, classical activation, which generates M1 macrophages, occurs in the presence of bacterial compounds (i.e. LPS) and Th1 typical cytokines, such as IFNγ (Martinez et al, 2008). On the other hand, the alternative M2 activation is dependent on Th2 typical cytokines, such as IL-4 and IL-13. M1 macrophages, defined as IL-12^{hi}, IL-23^{hi}, IL-10^{low}, are characterized by the production of effector molecules (reactive oxygen and nitrogen intermediates), Th1 and NK attracting chemokines (CXCL9,

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CXCL10), and pro-inflammatory cytokines (IL-1β, TNF, IL-6), which in turn promote the Th1 response, exerting a strong microbicidal and tumoricidal activity (Sica et al, 2008). However, M2 cells defined as IL-12^{low}, IL-10^{hi}, IL-1decoyR^{hi}, IL-1RA^{hi}, are considered to be involved in parasite containment and clearance and promotion of tissue remodelling together with Th2 cells. They express CCL17, CCL22 and CCL24, have immunoregulatory functions and promote tumour development and progression (Allavena et al, 2008a; Gordon & Martinez, 2010; Martinez et al, 2006 ; Sica & Mantovani, 2012).

M1 and M2 macrophages are distinct cells that exert opposite functions and respond to different stimuli (Mantovani et al, 2004). Analysing the gene expression profile of M1 and M2 macrophages showed that more than 1200 genes are selectively regulated. (Martinez et al, 2006) For example, molecules implicated in pathogen-recognition, in phagocytosis and co-stimulation are typical of M1 macrophages, conferring them with the capability to recognize pathogens through pattern recognition receptor, such as TLR (Biswas & Mantovani, 2010; Gordon & Martinez, 2010). Contrary to this, M2 macrophages express high levels of scavenger receptors and low levels of TLRs (Peiser & Gordon, 2001). Indeed, M2 macrophages are involved in the elimination of damaged self-components, which are recognized by the scavenger receptors and then eliminated through phagocytosis. Although phagocytosis is a key mechanism shared by both classically and alternatively activated macrophages, it leads to cytotoxicity and killing of the pathogen in M1 macrophages, whereas it favours repair, healing, regeneration and angiogenesis by M2 macrophages (Gordon & Martinez, 2010).

M1 and M2 macrophages have distinct features in terms of the metabolism of iron, folate and glucose (Zhu et al, 2015). Indeed, M1 stimuli are responsible for a shift towards the anaerobic glycolytic pathway. This effect mirrors the functional role of M1 macrophages, which are associated with acute inflammation and need to quickly exert their anti-microbial activity within the hypoxic tissue microenvironment. Anaerobic glycolysis is the process that best suits their rapid energy requirements (Biswas & Mantovani, 2012).

On the other hand, M2 macrophage-related functions, such as tissue remodelling, repair and healing, require a sustained supply of energy. Therefore, the metabolic pathway of M2 macrophages is preferentially based on fatty acid oxidation.

In addition, M1 macrophages express high levels of NOS2 and NO, which exert a key role for their microbicidal activity (MacMicking et al, 1997). In contrast, M2 macrophages do not produce NO, but express high levels of Arginase 1, which catalyses polyamines production necessary for collagen synthesis, cell proliferation, fibrosis and other tissue remodelling-related functions (Biswas & Mantovani, 2012).

Macrophage polarization is strictly regulated by a network of signalling molecules, transcription factors, epigenetic mechanisms and post-transcriptional regulators. IFNs and TLR signalling activate STAT1, whereas IL-4/IL-13 activate STAT6, skewing macrophage function towards the M1 or M2 phenotype, respectively (Sica & Bronte, 2007). IRF5 induction, which is downstream the TLR4 pathway activated by LPS, is responsible for the expression of cytokines (IL-12, IL-23, TNF) involved in sustaining Th1 and Th17 responses (Krausgruber et al, 2011). However, STAT6 activation induces M2-related genes, such as mannose receptor (MRC1), while activation of STAT3 induces a "M2-like" phenotype (see below), characterized by expression of genes such as IL-10, TGF-β, MRC1 (Gordon & Martinez, 2010; Mantovani & Sica, 2010; Pauleau et al, 2004).

NF-κB activation, dependent on TLR engagement, leads to the production of inflammatory mediators associated with M1-macrophages, but is also necessary for the resolution of inflammation and for M2 polarization of TAMs (Hagemann et al, 2008; Lawrence & Gilroy, 2007). Peroxisome proliferator-activated receptor gamma (PPARγ) agonists have been shown to induce M2-like differentiation (Bouhlel et al, 2007) and SHIP [SH2 (Src homology 2)-containing inositol phosphatase-1] was found to play a key role in balancing macrophage polarization, although recent evidence suggests that it may act indirectly through the regulation of IL-4 production by basophils (Kuroda et al, 2009).

Macrophage polarization could be influenced by M-CSF, the key regulator of monocyte differentiating processes (Solinas et al, 2009). M-CSF driven monocyte-to-macrophage differentiation is associated with a dramatic change in the transcriptome, whereas further stimulation with an M2 stimulus (IL-4) caused a smaller alteration in gene expression (Martinez et al, 2006). This apparently minor effect of IL-4 is due to the fact that treatment with M-CSF elicits functional responses which share some properties with M2 and are referred to as M2-like (Allavena et al, 2008a; Mantovani et al, 2005). "M2-like" macrophages are induced also by IL-10, TGF-β, glucocorticoid hormones, molecules that originate from apoptotic cells and immune complexes together with LPS or IL-1R ligands (Allavena et al, 2008a). They are defined as M2-like because they share some M2 features, such as the expression of high levels of mannose receptors and scavenger receptors but are distinct from them, for instance, in terms of the chemokine repertoire (Biswas & Mantovani, 2010).

Mononuclear phagocytes are highly plastic and flexible cells and M1 and M2 macrophages can, to some extent, be interconverted *in vitro* and *in vivo* (Biswas & Mantovani, 2010; Guiducci et al, 2005). Moreover, dynamic changes in macrophage activation occur in pathology: M1 macrophages are involved in the early and acute phases of inflammation, whereas M2 or M2-like macrophages are associated with resolution or smouldering chronic inflammation. At the present time, the mechanisms regulating these switches are still unclear. In particular, it remains to be defined whether the M1-M2 switch is due to recruitment of circulating precursors which polarized into the opposite way, or to the *in situ* re-education of cells (Martinez et al, 2009). The loss of equilibrium between M1 and M2 cell number may lead to pathological events: an M1 excess could induce chronic inflammatory diseases whereas an uncontrolled number of M2 could promote severe immune suppression (Allavena et al, 2008a).

1.4.2   TAMs as major effector of cancer-related inflammation

Macrophage infiltration, studied in mouse models of tumour carcinogenesis, begins very early during the pre-invasive stage of disease and increases progressively. TAM have mostly an M2-like polarization (IL-10^{hi}, IL-12^{low}, expression of mannose receptor and SR-A), as also indicated by transcriptional profiling (Biswas et al, 2006; Biswas & Mantovani, 2010; Sakai et al, 2008). In addition TAMs exert several M2-associated functions favouring cancer progression, invasion and metastasis, and suppressing anti-tumour immunity (Allavena et al, 2008a; Pollard, 2004; Talmadge et al, 2007). In agreement, low macrophage infiltration into the tumour mass correlates with the inhibition of tumour growth and metastasis development in different animal models (Hiraoka et al, 2008; Lin et al, 2006; Zeisberger et al, 2006).

The preferential M2 polarization is due to the absence of M1 orienting signals such as IFNγ or bacterial components in the tumour as well as to the expression of M2 polarizing factors. In particular, the infiltration of Th2 lymphocytes (driven by Th2-recruiting chemokines like eotaxin) has been reported in many tumours and they are a fundamental source of IL-4 and IL-13 cytokines (Solinas et al, 2009). In addition, neoplastic cells, fibroblasts and Treg produce TGF-β and IL-10. Moreover, gene modified mice and cell transfer experiments have confirmed the protumor function of myeloid cells and of their effector molecules. It has been demonstrated that when MMTV-PyMT mice, which spontaneously develop mammary tumours, were crossed with mice lacking monocytes/macrophages (op/op), the tumour growth and spread were significantly reduced (Lin et al, 2001). Accordingly, when co-cultured with tumour cells, macrophages secrete substances, like those that stimulate tumour cell proliferation (Allavena et al, 2008a; Balkwill, 2004; Pollard, 2004).

1.4.2.1 Regulation of transcriptional programs in TAMs

HIF-1 and NF-κB were found to be key regulators of the transcriptional program of TAMs (Mantovani et al, 2006). The reason why NF-κB activation in TAMs is related to tumour promotion is not completely clear (Lawrence, 2011). TAMs display defective NF-κB activation in response to various pro-inflammatory signals and this results in impaired macrophage-dependent inflammatory functions, such as the expression of cytotoxic mediators (i.e. NO) and cytokines (TNF, IL-1, IL-12p40). This defect was attributed to the over-expression of the nuclear p50 subunit of NF-κB, which leads to the generation of homodimers p50-p50, inhibiting NF-κB-dependent genes (Porta et al, 2009). TAMs from *p50*-deficient mice re-gained a pro-inflammatory (M1) phenotype, suggesting that a proper NF-κB activation is responsible for a "canonical" activation of macrophages (Biswas et al, 2006; Lawrence, 2011). In contrast, it was also shown that blocking NF-κB activation in TAMs isolated from early-stage tumours increased their tumour-suppressing activity (Hagemann et al, 2008). These studies suggest that NF-κB favours the M2-like phenotype in tumour associated macrophages.

The discrepancy concerning the role of NF-κB in TAMs may be explained by the dynamic changes occurring in the tumour microenvironment during the progression from early neoplasia towards advanced tumour stages. NF-κB activity in the inflammatory infiltrate may be progressively modulated by tumour cells themselves, producing several factors involved in NF-κB regulation (Allavena et al, 2008a; Sica & Mantovani, 2012).

Hypoxic stress is a common feature of solid tumours and it is principally regulated by the Hypoxia inducible transcription factor (HIF-1), whose activation in cancer cells favours their survival in a hostile environment (Allavena et al, 2008b). Indeed, it mediates the switch from aerobic to anaerobic metabolism, resulting in chronic acidification of the local microenvironment, leading to invasion and metastasis (Semenza, 2003; Vaupel, 2004). In addition hypoxic stress promotes angiogenesis, inhibition of apoptosis, up-

regulation of growth factors, such as PDGF, TGF-β, IGF-2, EGF, VEGF, and proteins involved in tumour invasion (Vaupel, 2004). Finally, it also contributes to cancer cell detachment, since it is responsible for down regulation of adhesion molecules and is able to influence the position and function of cancer and stromal cells, including TAMs by up-regulating the chemokine receptor CXCR4 (Salcedo et al, 1999). TAMs are therefore recruited into hypoxic sites and are able to adapt to hypoxia through the activation of hypoxia inducible transcription factors HIF-1 and HIF-2, which induce the expression of pro-angiogenic genes as well as glycolytic enzymes (Talks et al, 2000).

1.4.2.2   TAMs and angiogenesis

Initiation of angiogenesis in an important early event in tumour progression (Ruoslahti, 2002). Indeed, tumours cannot grow beyond a certain size without recruitment of endothelial cells that generate new vessels to supply nutrients and oxygen for tumour cell survival. Compared with the vasculature in normal tissues, the tumour vessels differ both in morphology and functionality. They are tortuous and leaky; their diameter is irregular and their walls are thin. Blood flow through the tumour capillaries is frequently sluggish, and at times, might be stationary or even experience a reversal in the direction of flow (Tozer et al, 2005).

VEGF is the primary, but not the only, angiogenic factor released by tumour cells, and is involved in the "angiogenic switch" that can occur at various stages of tumour progression, depending on the tumour type and the microenvironment. Many other factors are involved, including PDGFβ, bFGF, angiopoietins and CXCL12 (SDF-1) (Du et al, 2008). TAMs have a profound influence on the regulation of tumour angiogenesis, expressing various pro-angiogenic molecules, such as VEGF, bFGF, TNFα, IL-1β, and various chemokines and proteases (Murdoch et al, 2008). Pre-clinical studies demonstrated that TAMs positively correlated with microvascular density (MVD) (Leek et al, 1996; Takanami et al, 1999), while depletion of monocytes by clodronate treatment results in lower TAM infiltration and angiogenesis (Murdoch et al, 2008).

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As mentioned before, hypoxia activates in these cells a specific pro-angiogenic program (Mantovani et al, 2002). Low oxygen conditions promote HIF-1 and HIF-2 expression with subsequent over-expression of pro-angiogenic molecules. Hypoxia exerts a crucial role in the up-regulation of gene transcription in TAMs, promoting VEGF expression (Burke et al, 2003; Murdoch et al, 2005). Of note, TAMs express VEGF almost exclusively in avascular and perinecrotic areas, as observed for instance in human breast carcinomas (Murdoch et al, 2008). The importance of HIF-1 was underlined by the observation that in hypoxic regions, the ablation of this transcription factor leads to an impaired motility and cytoxicity of macrophages (Cramer et al, 2003). Among chemokines, hypoxia tightly regulates the expression of CXCL12, a HIF-1-dependent and potent chemoattractant for endothelial cells, and of its receptor CXCR4. In addition to CXCL12, TAMs release other chemokines involved in the angiogenic processes such as CCL2, CXCL8, CXCL1, CXL13 and CCL5 (Balkwill, 2004; Murdoch et al, 2008). For instance, levels of CXCL5 and CXCL8 were associated with increased neo-vascularization and inversely correlated with survival (Balkwill, 2004; Mantovani et al, 2004; Strieter et al, 2004).

TAMs play also a role in peritumoral lymphoangiogensis, an initial step for the spreading of tumour cells. The process is mediated by a number of factors including VEGF-C, VEGF-D and their receptor VEGFR3 (Ji, 2012; Schoppmann et al, 2002), all secreted or expressed by TAMs (Ji, 2012; Schoppmann et al, 2002). In addition, it has been shown that VEGF-A increases lymphangiogenesis via recruitment of monocytes.

1.4.2.3   *TAMs and cancer cell dissemination*

The major cause of death in cancer results from therapy resistant metastases (Fidler, 1999). Stephen Paget's conclusion in the late 19th century (Paget, 1989) that the metastatic process depends on cross-talk between selected cancer cells (the "seed) and a specific organ microenvironment (the "soil") is still valid, and is experimentally confirmed (Fidler, 2003; Talmadge & Fidler, 2010). Tumour metastasis is a complex multistep process,

during which malignant cells spread from the primary tumour site to secondary distant organs. The different steps of cancer cell dissemination can be subdivided into local invasion, entry into the bloodstream (intravasation), survival in the bloodstream, extravasation and colonization (Nguyen et al, 2009).

Mesenchymal, endothelial and immune cells are required to form an appropriate microenvironment for tumour progression (Joyce & Pollard, 2009). Immune cells, particularly macrophages, neutrophils, T-lymphocytes, and NK cells, are major sources of proteases that degrade the host tissue, allowing cancer cells to disseminate. The set of proteolytic enzymes found in tumour microenvironment comprises matrix metalloproteases, serine proteases and cysteine proteases (i.e.cathepsin) (Decock et al, 2011; Egeblad & Werb, 2002; Lynch & Matrisian, 2002). Matrix proteases play essential functions in physiological conditions as active regulators of post-natal tissue development and remodelling (Alford & Hankenson, 2006). In addition, they are important for tissue repair in response to injury, and regulate cancer progression modulating the tumour microenvironment, particularly the infiltration of leukocytes (Page-McCaw et al, 2007).

TAMs contribute to cancer cell dissemination by releasing several motility factors and enzymes involved in degradation of the ECM such as matrix metalloproteinase 2 (MMP2) and MMP9, urokinase plasminogen activator, tissue-type plasminogen activator, and cathepsin (Gocheva et al, 2010). One of the main factors significantly involved is TNF: co-colture of neoplastic cells with macrophages enhances invasiveness of malignant cells through TNF-dependent MMP induction in macrophages (Hagemann et al, 2004).

Giavazzi and colleagues demonstrated the IL-1β cytokine produced by TAMs, induces a higher development of metastases in a mouse melanoma model (Giavazzi et al, 1990). In addition, in a genetic model of breast cancer in monocyte-deficient mice, tumours developed normally but, in the absence of the macrophage-produced EGF, they were unable to form pulmonary metastases (Pollard, 2008).

TAMs are important players of tumour progression and metastatic colonization through the cross-talk with tumour cells (Brennecke et al, 2014). For instance, macrophages play a crucial role in conferring an invasive phenotype to epidermal keratinocytes from Snail transgenic mice (Du et al, 2010).

Cytokines produced by TAMs can also play a role in metastatization. In breast cancer, EGF secreted by TAMs increases migration and invasion of neighbouring breast cancer cells, which express high levels of EGF receptor (EGFR). On the other hand, cancer cells secrete high levels of M-CSF, a main chemoattractant for TAMs which expresses the cognate receptor MCSF-R. Therapies aiming at inhibiting this cross-talk by blocking MCSF-R or/and EGFR were shown to be successful (Goswami et al, 2005). Macrophages and their cross-talk with tumour cells are mandatory for tumour cell migration, regardless of the factors inducing cell invasion (e.g. CXCL12) (Brennecke et al, 2014).

1.4.2.4   TAMs and immune suppression

Besides the effect on tumour growth and dissemination, TAMs have also the potential to suppress the adaptive immune response through different mechanisms including poor antigen-presenting activity and inhibition of T cell proliferation, leading to cancer immune evasion (Dunn et al, 2004). As mentioned above, M2-like TAMs are characterized by an immunosuppressive phenotype, with production of high levels of IL-10 and TGF-β and reduced expressions of immune-stimulatory molecules such as IL-12 (Biswas et al, 2006; Biswas & Mantovani, 2010; Hagemann et al, 2008). It has been shown that IL-10, alone or in concert with IL-6, is responsible for the up-regulation of macrophage B7-H4 expression, a molecule implicated in the suppression of tumour-associated antigen-specific T cell immunity (Kryczek et al, 2006).

In this regard, TAMs are also able to influence the adaptive immune response, suppressing the potential anti-tumoral activity of B and T cells (Solinas et al, 2009). In addition, dendritic cells in the tumour microenvironment were observed to be immature and therefore not able to efficiently prime T cells that become anergic. DC maturation is

blocked because of the secretion of various cytokines by several tumour-associated cells, including TAMs, which express high levels of IL-10.

Furthermore, TAMs secreted chemokines such as CCL17 or CCL22, that preferentially attract Th1, Th2 and Treg lymphocytes with defective cytotoxic functions, or such as CCL18, induced in normal macrophages by Th2 cytokines (IL-4; IL-13; IL-10), that recruit naïve T cells which become anergic in contact with M2 macrophages and immature dendritic cells (iDC) (Banchereau & Palucka, 2005; Mantovani & Sica, 2010). CCL18 has been also identified as the most abundant chemokine in the ascites fluid of human ovarian carcinoma (Schutyser et al, 2002).

1.4.2.5 TAMs and anti-cancer therapies

As we discussed above tumour-associated macrophages favour neoplastic cells during tumour development, invasion and spread to distant sites (Solinas et al, 2009). Therefore these cells may certainly be considered as an attractive target for novel anti-cancer therapies. Thus, it is important to ask the question that "if we block macrophages, will we actually disturb tumour progression in patients?"

As a matter of fact high levels of TAM infiltration generally correlates with poor outcome for patients (Bingle et al, 2002; Mantovani et al, 2008b; Pollard, 2004; Qian & Pollard, 2010), but a few exceptions to this finding are also reported. For instance in colorectal cancer (CRC) there are contrasting results such that TAM density is associated with positive or negative outcomes (Erreni et al, 2011; Forssell et al, 2007; Ohno et al, 2003). Macrophage subsets might have distinct roles, as observed in lung adenocarcinoma, where the number of CD204+ TAMs showed a strong association with poor patient outcome, while the CD68+ TAMs population did not (Ohtaki et al, 2010).

The concept that not only the number and the presence of specific cell subsets, but also the localization of infiltrating cells might have specific functions and predictive values is increasingly emerging (Brennecke et al, 2014). Accordingly, peritumoral density of TAMs expressing co-stimulatory molecules (CD80 and CD86) was associated with better patient

survival in CRC, whereas the same cell population within the tumours themselves did not have predictive value (Sugita et al, 2002). Thus TAMs exert complex roles on growing tumours affecting different aspects of tumour progression, namely tumour cell proliferation and survival, angiogenesis, tumour dissemination and resistance to therapies.

Within a tumour, the heterogeneous microenvironment differentially influences infiltrated macrophages and this clearly shows the necessity of identifying the correct TAMs on target for the generation of new therapeutic molecules (Solinas et al, 2009). Obviously, the best target would be a protein expressed or over-expressed only by TAMs and neither by resident macrophages of distant healthy tissues nor by M1 cells, which are important to remove pathogens, and could take part in anti-cancer actions.

Interestingly, observations come from studies performed with chemokines and chemokine receptors as anti-cancer targets. For instance, in a breast cancer murine model, malignant cells recruit macrophages via CCL5 and treatment with CCL5 receptor antagonists leads to a decreased number of infiltrating macrophages associated with a significantly reduced tumour size (Robinson et al, 2003).

Recruitment of monocytes into the tumour microenvironment is dependent on CCL2 and growth factors, such as M-CSF and VEGF, which are also responsible for M2/M2-like skewing of macrophages. Inhibition of MCSF-R has an anti-angiogenic and anti-metastatic effect in acute myeloid leukemia and melanoma models (Qian et al, 2011).

The anti-tumoural drug Trabectidin (Yondelis) derived from mourine tunicates has immunomodulatory properties on mononuclear phagocytes. Our group has recently demonstrated that this drug show a selective cytotoxic effect on monocytes and macrophages, including TAMs, while sparing the lymphoid subset (Germano et al, 2013); moreover, at subcytotoxic concentrations, Trabectidin significantly reduced the production of CCL2 (Allavena et al, 2005). CCL2 has also been identified as a fundamental regulator of prostate cancer growth and metastasis and its inhibition with anti-CCL2 antibodies, in combination with docetaxel, induces a significant tumour regression (Loberg et al, 2007).

Inhibitors of the CCL2/CCR2 axis have also been shown to exert a significant anti-tumoral activity in a model of pancreatic cancer (Sanford et al, 2013)

Thalidomide, linomide, pentoxifyline and genistein and other anti-angiogenic factors have been demonstrated to inhibit macrophage recruitment and to reduce tumour size (Vukanovic & Isaacs, 1995). Moreover, it has been shown that anti-VEGF-A neutralizing antibodies reduce the development of new blood and lymphatic vessels and decreased incidence of lymphatic and pulmonary metastasis in an orthotopic breast tumour model (Whitehurst et al, 2007). As already mentioned, VEGF also promotes macrophage recruitment into tumours and Dineen and colleagues demonstrated that specific inhibition of VEGFR2 significantly decreases macrophage infiltration into orthotopic pancreatic tumours (Dineen et al, 2008).

Other efficient anticancer molecules are MMP inhibitors such as the bisphosphonate zoledronic acid (Giraudo et al, 2004). In murine models of genetic carcinogenesis, suppression of MMP-9 secretion by TAM results in a slower tumour growth (Melani et al, 2007).

Given that macrophages spontaneously infiltrate tumours, recent studies used these cells as natural vectors to deliver therapeutic molecules to the neoplastic site. Intratumoral injection of macrophages transduced with a recombinant adenoviral vector expressing IL-12 led to the reduction of primary tumour growth and lung metastases in a preclinical model of metastatic prostate cancer, with an higher number of tumour infiltrated CD4+ and CD8+ T cells compared with the controls animals (Satoh et al, 2003). Another fascinating approach is targeting stromal-cancer cell interactions with siRNA (Aharinejad et al, 2009). Macrophages were also used to enhance the immune response or to potentiate chemotherapy specificity. Carta and colleagues engineered a murine macrophage cell line which strongly augmented the production of IFNγ (Carta et al, 2001).

A contribution of the immune system to the anti-tumour effects of conventional chemotherapy has been suggested (Zitvogel et al, 2008). Dying cells can secrete proteins,

termed DAMPs (damage-associated molecular patterns) that activate innate immune cells (Green et al, 2009; Zitvogel et al, 2008). For example, late apoptotic cells express SIN3A-associated protein 130 (SAP130), a ribonucleoprotein which binds to the macrophage-inducible C-type lectin receptors (MINCLE). In addition it has been shown that inhibition of MINCLE interrupts the release of pro-inflammatory cytokines by macrophages and also blocks neutrophil recruitment (Green et al, 2009; Yamasaki et al, 2008).

1.4.2.6   Macrophage reprogramming

The so-called macrophage re-education, which is based on the idea of plasticity of macrophages, is the "holy grail" of macrophage targeting in cancer treatment (Mantovani & Sica, 2010; Sica & Mantovani, 2012). Indeed, polarized macrophages are able to revert or at least modify their polarization upon treatment, both *in vivo* and *in vitro*. In a large clinical study of ovarian cancer, IFN-γ was found to induce a phenotype switch in TAMs, leading to the activation of tumoricidal properties of these cells (Allavena et al, 1990). In a model of pancreatic ductal adenocarcinoma, CD40 agonist antibodies promoted the repolarization of TAMs, which started to express high levels of M1 related proteins (MHC class II and CD68) and therefore to have an anti-tumoral effect (Beatty et al, 2011). Thus, even if therapeutic macrophage targeting has only recently started to be considered as an effective approach, it seems to be promising and the identification of mechanisms and molecules associated with macrophage plasticity and polarized activation provides a basis for macrophage-centered diagnostic and therapeutic strategies.

The balance between M1 and M2 polarized macrophages is a fundamental aspect in anti-cancer treatment. Activation of TLR stimulates M1-polarized macrophages, inducing activation of a pro-inflammatory program (Krieg, 2006). Synthetic CpG oligodeoxynucleotides (CpG-ODN), detected by TLR9, demonstrated anti-tumour effects in many pre-clinical models even though they do not seem to work with large established tumours (Krieg, 2004). Moreover, when CpG-ODN have been added to chemotherapy agents, both the response and the survival were significantly increased (Manegold et al,

2008). Rauh and colleagues highlighted the importance of the already mentioned SHIP1 in reverting M1 *versus* M2 polarized macrophages. *SHIP1* deficient mice showed skewed development towards M2 macrophages (Rauh et al, 2004) and thus pharmacological modulators of this phosphatase are under investigations (Guiducci et al, 2005).

Hagemann T. et al. (2008) have shown that the immunosuppressive phenotype in TAMs is dependent on the type of activation of NF-κB, which is modulated by malignant epithelial cells (Hagemann et al, 2008). Targeting IKKβ activity in TAMs isolated from tumours resulted in NF-κB inactivation, leading to the conversion from a pro-tumour to an anti-tumour activity, typical of M1 macrophages. Indeed, the production of IL-10, TNF and expression of Arg-1 were reduced, whereas the production of IL-12 and nitric oxide synthase were increased. Moreover, TAMs showed enhanced MHC class II expression and, when transferred into ovarian cancer bearing mice, they promoted NK cell recruitment at the tumour site and a significant decrease in tumour burden.

IFN-γ treatment was used to switch immunosuppressive TAMs into M1-like cells. Upon treatment with IFN-γ, TAMs expressed high levels of IL-12 and CD68 and low levels of IL-10 and IL-T3, enhancing the proliferation of CD4+ T cells and the cytotoxic properties of CD8+ T cells. Moreover, they secreted reduced levels of mediators promoting suppressive T cell recruitment (CCL-18) and favouring tumour growth and invasion (VEGF, MMP9). In addition, it was demonstrated *in vitro* that, in the presence of ovarian ascites, IFN-γ skewed monocyte differentiation from TAM-like cells to M1 macrophages (Duluc et al, 2009).

From what has been described so far, it is apparent that the tumour microenvironment affects profoundly the behaviour of host infiltrating cells. To address the role of the tumour microenvironment on the shaping of TAMs, human monocytes from peripheral blood were differentiated to macrophages in the presence of tumour-conditioned growth medium (TC-MΦ) and then submitted to global gene profiling (Solinas et al, 2010). The results

identified a series of up-regulated genes in TC-MΦ compared to resting monocytes (Figure 6) (Solinas et al, 2010). Interestingly the global gene profiling comparison of TC-MΦ, M1 and M2 macrophages and TAM revealed that some of the up-regulated genes in TC-MΦ were similarly expressed and regulated in M2 macrophages and TAM, but not in M1 polarized macrophages. These results further support the hypothesis that cancer cells can induce the M2-like polarization observed in TAM. In particular, among the genes differentially up-regulated in TAM and M2 polarized macrophages is FN1.

FN1 is a soluble glycoprotein that mediates a wide variety of cellular interactions with the extracellular matrix and plays important roles in cell adhesion, migration, growth and differentiation (Mosher & Furcht, 1981)

1.4.2.7   *Fibronectin 1 and its oncofoetal isoforms*

It is known that in transformed cells and in malignancies, the splicing pattern of Fibronectin pre-mRNA becomes altered (Kaczmarek et al, 1994), leading to an increased expression of oncofoetal isoforms (Oyama et al, 1989; Oyama et al, 1990) which are usually highly present both in fetal and human tumour tissues but seldom in the normal counterpart. Oncofetal proteins such as Corion Embryonic antigen CEA, α-fetoprotein, glypican-3, and nucleophosmin seem to be of embryonic origin, (Lee et al, 2008) and it has been widely observed that they are shared between the processes of embryogenesis and the one of tumorigenesis (Hendrix et al, 2007). Aggressive tumour cells and embryonic progenitors overlap in terms of some properties and thus it might be that also tumour-infiltrating stromal cells acquire foetal characteristics when activated by neoplastic signals (Hendrix et al, 2007).

Among the several isoforms of FN1 described, the expression of some of them was analysed in TC-MΦ, namely the Extra Domain-A (ED-A) and ED-B and Migration Stimulating Factor (MSF).

ED-A and ED-B (Gutman & Kornblihtt, 1987; Schwarzbauer et al, 1987; Zardi et al, 1987) are the most well-known oncofetal isoforms of fibronectin. They are highly

expressed in many tumours and seem to promote cell spreading and angiogenesis (Kaczmarek et al, 1994; Labat-Robert, 2002; Midulla et al, 2000). In particular, ED-B is produced during active tissue remodelling, such as in wound healing and in tumour angiogenesis processes, giving rise to a prominent perivascular expression pattern (Ffrench-Constant et al, 1989). This accumulation around neovascular structures has been extensively demonstrated in studies on many different tumor types, in particular, on invasive ductal carcinoma (Kaczmarek et al, 1994) and brain tumours (Castellani et al, 2002), as well as in ocular angiogenesis (Birchler et al, 1999). In addition, Mhawech *et al.* showed that head and neck tumours with a positive staining for ED-B had a trend to a significant lower overall survival of patients (Mhawech et al, 2005).

A third oncofoetal FN isoform has been cloned in 2003 by Schor and colleagues and it is known as Migration Stimulating Factor (MSF). MSF differ from the other two oncofoetal full length isoforms since it is identical to their 70-kDa N-terminus but terminates in a unique 10 amino acid long sequence. As described, in TC-MΦ, MSF is more express than ED-A and ED-B isoforms and so we focused our attention on the study of this "truncated isoform" and its involvement in tumour microenvironment.

1.5   Migration stimulating factor (MSF)

MSF, which is the focus of this thesis, is a truncated isoform of human fibronectin 1, expressed during foetal life (Schor et al, 2003). *Ex vivo* studies have shown that both MSF messenger RNA and protein are expressed by fibroblasts, keratinocytes, and vascular endothelial cells in foetal skin, but not by the majority of these cells in the healthy adult skin (Schor, 1994; Schor et al, 2003). In addition, Schor and colleagues a

lso demonstrated that MSF could be constitutively expressed *in vitro* by foetal fibroblast and fibroblast from cancer patients, by tumour cells (carcinoma, melanomas, and gliomas) and by tumour-associated stromal cells, but not by healthy adult cells (Schor et al, 1988a; Schor et al, 1988b). Recently Solinas et al. found that M2 polarized macrophages, but not

M1 macrophages, exposed to the TME express MSF (Solinas et al, 2010). This observation suggests that MSF may be a good candidate marker of M2-like TAMs.

Family	Symbol	Description	Fold Increase
C-type lectin R	MRC1	Mannose receptor/CD206	44
	CD209	DC-SIGN	3.6
	CD302	DCL-1	3
	CLEC10A	MGL/CD301	2.5
Fcγ receptors	FCGR2B	CD32	10
	FCGRT	Fc for IgG transporter	4
	FCGR1A	CD64	2.5
Scavenging receptors	MSR1	Scavenger receptor, SR-A	9
	STAB1	Stabilin1/SPARC-R	6
	CD163	CD163	5
	SCARB1	Scavenger R, class B	3
ECM molecules	GPNMB	OA	83
	SPP1	OPN	26
	F13A1	Factor XIII A1	11
	FN1	Fibronectin	7
Adhesion molecules and related	PMP22	Myelin-associated protein	22
	SIGLEC1	Sialoadhesin 1	12
	SLAMF8	CD2 family member	11
	ITGB5	β5 integrin	3.5
	CD81	CD81	3
	SIGLEC7	Sialoadhesin 7	2.7
	SELPLG	P selectin ligand	2.5
	ITGA4	α4 subunit, VLA4	2.1
Enzymes	ADAMDEC1	Decysin	7
	CPM	Carboxipeptidase	6
	MMP9	MMP9	6
	ADAM28	ADAM28	4
	PLAU	uPA	4
	MMP2	MMP2	3
MHC molecules and related	HLA-DMA	HLA-DMA	5
	CIITA	MHC II, transactivator	3
	CTSC	Cathepsin C	9
	CTSD	Cathepsin D	4
	CTSB	Cathepsin B	3
	CTSK	Cathepsin K	2.01
Protein trafficking	VPS45	Vacuolar sorting protein	5
	VPS13B	Vacuolar sorting protein	3.01
	LAMP1	Lysosomal protein 1	3
	LAMP2	Lysosomal protein 2	3
Miscellaneous	SEPP1	Selenoprotein 1	95

Human monocytes were stimulated for 72 h with tumor-CM. Results (mean of three different donors) are fold-increase relative to unstimulated monocytes.

Figure 6. Gene expression analysis of TC-MΦ. Genes upregulated in TC-MΦ compared to resting monocytes. Transcriptional profile has been analysed after 72 hrs of culture. Taken from Solinas et al. (2010)

1.5.1 MSF structure and functions

Human MSF is a soluble, genetically truncated isoform of human FN1 (EMBL AJ2535086) (Schor et al, 2003). The expression of MSF protein is controlled at the post-transcriptional level by a two-step mechanism. The primary 5.9 Kb MSF transcript is generated from the *fibronectin* gene by read-through mechanisms between exons III-1a and

III-1b followed by intra-intronic cleavage. The 5.9 kb MSF pre-messenger has an AU-rich instability element in its 3'-UTR that promotes rapid degradation of the transcript when sequestered in the nucleus. In cells expressing MSF protein the pre-messenger RNA is further cleaved to produce a 2.1 kb mature mRNA that is rapidly transported to the cytoplasm for translation (Kay et al, 2005).

The mature mRNA has a 1,941 bp long sequence identical to the 5' coding sequence of FN1 up to exon III-Ia. In addition it contains a 195 bp long intron-derived 3'-sequence, immediately contiguous with exon III-1a and containing a 30 bp in-frame sequence coding for the MSF-unique 10-amino acids long peptide (VSIPPRNLGY). Finally there is a 165 bp 3'-UTR containing five in-frame stop codons and terminating with a poly (A) tail (Kay et al, 2005). MSF protein is 657 amino acid long and has a predicted molecular weight of 70 kDa. Thus the primary amino acidic sequence of MSF is identical to the N-terminal region of FN1 up to and including the amino-acid sequence encoded by exon III-1a, with the addition of the MSF-unique 10-amino acid long peptide VSIPPRNLGY (Kay et al, 2005) (Figure 7).
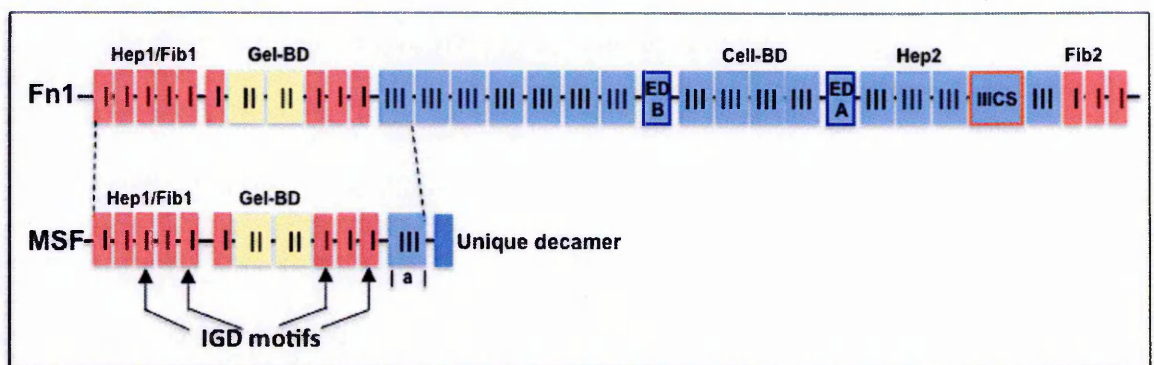
Figure 7. Comparison of the domain structure of MSF and FN1. MSF protein is identical to the N-terminal region of FN1 and terminates with a 10-amino acid long sequence (VSIPPRNLGY) not present in any previously identified fibronectin isoform. The black arrows show the IGD motifs that affecs fibroblast and endothelial cell migration. Figure adapted from Schor AM and Schor SL, (2010).

Both developmental and physiological signals, such as TGF-β, control the transcription and translation of MSF mRNA (Borsi et al, 1990; Keski-Oja et al, 1988; Wrana et al, 1991). Kay showed that TGF-β increases 3'-UTR processing of the nuclear 5.9 kb MSF

mRNA. However, at the same time, it promotes the synthesis of 5.9 kb MSF mRNAs, leading to the preferential increase of cytoplasmic levels of 2.1 kb MSF mRNA after TGF-β treatment (Kay et al, 2005).

Truncated fibronectin isoforms have also been detected in cDNA libraries from goldfish and rainbow trout, as well as from mouse and human liver, prostate, ovary, brain and spleen (Liu et al, 2003). Unpublished results mentioned by Schor & Schor (Schor & Schor, 2010) report the existence of a murine MSF protein expressed during foetal development, and a similar generation of MSF mRNA has been observed in Zebrafish embryo (Zhao et al, 2001).

MSF is a potent motogenic protein, involved in the stimulation of the migration of epithelial cells (normal and tumoural), adult-derived skin fibroblasts, vascular endothelial cells and pericytes (Houard et al, 2005; Hu et al, 2009). These cells show a typical bell-shape dose response with half-maximal activity at 0.1-1.0 pg/ml, corresponding to femtomolar concentrations.

MSF also induces the migration of non-epithelial tumour cells and shows other activities that characterize tumour progression, such as the up-regulation of hyaluronan (HA) synthesis, proteolysis, and induction of angiogenesis (Ellis et al, 1992; Houard et al, 2005; Hu et al, 2009; Schor & Schor, 2010; Schor et al, 2003; Schor et al, 1989).

The IGD tripeptide motif (isoleucine-glycine-aspartic acid) is a highly conserved feature of fibronectin type I modules to which no biological functionality was initially ascribed (Hynes, 1986). MSF contains four such IGD motifs (Figure 7). *In vitro* mutagenesis studies have indicated that the stimulation of fibroblast migration by MSF is mediated by two IGD motifs present in type I modules I-7 and I-9, whereas all four IGD motifs (I-3; I-5; I-7; I-9) affect endothelial cell migration (Ellis et al, 2010). Critically, small IGD-containing synthetic peptides mimic all MSF bioactivities, such as the stimulation of cell migration, endothelial cell activation and angiogenesis (Schor & Schor, 2010). IGD motifs are cryptic in fibronectin, for this reason FN1 does not show the

peculiar activities displayed by MSF (Millard et al, 2007; Vakonakis et al, 2009). MSF also maintains the proteinase activity exerted by FN1, dependent on the presence of the putative zinc-binding site HEEGH located in type I module I-8 (Unger & Tschesche, 1999) (Houard et al, 2005). Interestingly the proteinase activity exerted by MSF seems to mediate the motogenic response of a breast cancer cell line (MCF7) (Houard et al, 2005).

MSF induces endothelial cell activation *in vitro*, with appearance of a network of cells characterized by a sprouting phenotype (Schor et al, 2001; Schor et al, 1983). In addition MSF induces angiogenesis in rats and pigs implanted with collagenous matrices soaked with MSF (Schor & Schor, 2010).

1.5.2   Regulation of MSF expression

The ECM plays an important role on regulation of MSF expression. Schor *et al* demonstrated that normal adult fibroblasts, normally not producing MSF, can be induced to express the protein by a transient treatment with TGF-β, but only when they are growing on a "wounded" substratum *in vitro* (such as a denatured type I collagen or fibrin). This expression is persistent for the entire subsequent lifespan of the treated fibroblasts, even if cultured in the absence of TGF-β and irrespective of the nature of the substratum. Moreover, as a consequence of a second transient exposure to TGF-β, MSF expression may be switched off, provided that the cells are growing on a "healthy" matrix of native type I collagen. Thus, this "on/off" switch is repeatable and dependent on the concerted action of TGF-β and the presence of an appropriate matrix (Schor et al, 2012).

Epigenetic studies demonstrate a persistent switch-on of MSF expression following a transient exposure of normal adult fibroblast to 5'-azacytidine, a pharmacological agent inducing changes in gene expression through CpG island demethylation (Schor & Schor, 2010). Consistent to what is described above, this induced expression may be switched-off by a subsequent exposure of activated cells to TGF-β when grown on a native collagen substratum. These observations indicate that the nature of the substratum is a critical

component in the regulation of MSF expression. Thus the effects of TGF-β on target cells are dependent on the nature of the ECM. This is true also in the case of fibroblast migration, that is manifested by cells adherent to native, but not denatured type I collagen substratum. Native collagen may accordingly be considered to provide a "permissive" substratum, whereas denatured collagen is "non-permissive" (Schor & Schor, 2010; Schor et al, 1999; Schor et al, 2003).

Besides TGF-β, MSF bioactivities can be modulated by various soluble factors, including neutrophil gelatinase-associated lipocalin (NGAL), produced by "resting" keratinocytes (Ellis et al, 1992; Jones et al, 2007). In addition neutralizing anti-MSF antibodies were described that can induce apoptotic death of target endothelial cells (Schor et al, 2003). This suggest that MSF could also act as an autocrine survival factor.

Finally, there is evidence that MSF can be expressed during tumour formation in human cancer-associated myofibroblasts (Carito et al, 2012). This work indicate that overexpression of MSF is sufficient to confer myofibroblastic differentiation by increased TGF-β signalling. In addition, MSF activates NF-κB, resulting in the onset of autophagy/mitophagy, thereby driving glycolytic metabolism (L-lactate production) in the tumour microenvironment (Carito et al, 2012). Thus, MSF can participate in the metabolic remodelling of the tumour microenvironment by reprogramming cancer-associated fibroblasts toward a glycolytic metabolism.

The potential clinical relevance of these findings is highlighted by the observation that constitutive MSF expression by foetal and cancer patient fibroblasts may be persistently switched off by the exposure to TGF-β of cells growing on a native collagen substratum (Schor et al, 2012). The possibility to down regulate the potentially deleterious MSF expression by pharmacological intervention suggests that it may be possible to develop novel therapeutic strategies blocking MSF expression in particular clinical contexts.

1.5.3 MSF as a novel target for clinical intervention

A systematic assessment of MSF expression in human tumours is still lacking, however the data available so far indicate that more than 80% of common human tumours are characterized by MSF expression (Schor & Schor, 2010). Recent data showed that high MSF expression in tumours and tumour-associated stromal cells correlates with a poor survival in patients with breast cancer and salivary gland tumour (Schor & Schor, 2010). These observations suggested that MSF could represent a new marker associated with neoplastic transformation. Thus, assessment of MSF expression could provide independent prognostic information. Using an unbiased proteomic screening, MSF has been identified as a critical angiogenic factor driving oesophageal cancer progression (Hu et al, 2009). This evidence supports the role of MSF in cancer pathogenesis. Yoshino *et al.* (2007) have additionally reported that MSF expression is induced also by the exposure of a bronchioloalveolar carcinoma cell line to the tobacco carcinogen benzo[a]pyrene (Yoshino et al, 2007).

As mentioned, tumour-conditioned culture medium was able to induce MSF expression in TC-MΦ (Solinas et al, 2010). TC-MΦ display a gene expression profile that resembles M2 polarized macrophages and TAMs (Solinas et al, 2010). These observations suggest that MSF could be a novel molecule associated with the M2 and M2-like polarization and for this reason could represent a new marker for identification of TAMs in human tumours (Solinas et al, 2010).

The various facets of MSF expression and bioactivity outlined above suggest that MSF may be considered as a novel target for clinical intervention. Such intervention strategies may involve agents that modulate its reversible expression (including siRNA therapy) and/or abrogate manifestation of its bioactivities (neutralizing antibodies and/or pharmacological agents). The clear influence of microenvironmental parameters in modulating both its expression and manifestation of its bioactivities offer additional potential means of clinical manipulation.

2 AIM OF THE STUDY

Migration Stimulating Factor is an oncofetal isoform of human fibronectin 1 known for long time, but only recently its production has been found selectively up-regulated in M2 and M2-like polarized macrophages (Solinas et al, 2010).

Tumour-associated macrophages are major components of the host cell infiltrate of tumours and are characterized by a M2-like phenotype (Mantovani & Allavena, 2015). Strong epidemiological and experimental evidences have demonstrated that TAMs profoundly affect the tumour milieu expressing several pro-tumoural functions (e.g. tumour proliferation and invasion, angiogenesis, ECM degradation) and eventually promoting tumour progression and resistance to therapy (Solinas et al, 2009). Importantly, high levels of TAM are associated with bad prognosis in many tumours (Solinas et al, 2009).

These findings suggest that MSF could be a potential marker for tumour progression associated to M2-like polarization of tumour infiltrating macrophages and a promising target for anti-cancer therapy. Thus aim of this project was:

a) to generate reagents (recombinant protein and antibodies) useful to identify MSF in human tumours and in tumour-bearing subjects.

b) to characterize the biological properties of human MSF.

3 MATERIALS AND METHODS

3.1 Reagents

The list of primers used to amplify MSF mRNA by PCR is reported in Table 1. Two sets of primers were used: CL1 amplified a 771 bp fragment in the 5'end of both MSF and FN1, while CL2 produced a 1448 bp fragment specific for the 3' end of MSF. The sequence of forward and reverse primers for Real-time quantitative PCR (RT-PCR) is also reported in Table 1.

Table 1. Primers used to analyze MSF expression in TC- MΦ				
Set of primers		Restriction site and Primers (5'→3')	bp[a]	Tm[b] (C°)
CL1	Fw	5'- (BamHI) CGGGATCCCGCAAACTTGGTGGCAACTTGCCT-3'	771	68
	Rev	5'-GTCCTGATCGTTGCATCT-3'		48
CL2	Fw	5'-GAGAAGCCCTACCAAGGCTGGATGATGGTAGATTGTACTTG-3'	1448	69
	Rev	5'- (NotI)ATAAGAATGCGGCCGCTAATTGTTGGCTGAAA AGCTGGGAA-3		68
RT-qPCR	Fw	5'-GCATTGCCAACCTTTACAGAC-3'	177	60
	Rev	5'-TTTCTGGGTGGGATACTCAC-3'		

[a] Amplicon size
[b] Melting temperature

Human Fibronectin 1 (FN1) was purchased from Calbiochem (Merck, Milan, Italy); rabbit polyclonal antiserum anti-FN1 (ab299) was purchased from Abcam (Cambridge, UK); mouse anti-FN1 antibody (MAB 1892) recognizing the gelatin-binding domain of fibronectin (Gel-BD; clone IST-10) was purchased from Millipore (Merck, Milan, Italy). Mouse mAb recognizing histidine tail was obtained from Invitrogen (Milan, Italy). Fetal calf serum (FCS) was from Euroclone (Milan, Italy); RPMI-1640 and Dulbecco Modified Eagle's Medium (DMEM) from LONZA (Euroclone, Milan, Italy). Phosphate buffered saline (PBS) with calcium and magnesium (PBS^{+/+}) was from Biosera (Biotecna, Milan,

Italy), while PBS without calcium and magnesium (PBS$^{-/-}$) was from LONZA (Euroclone), as well as trypsin used for cell culture; geneticin (G418) was from Sigma-Aldrich (Milan, Italy).

The synthetic peptide specific for human MSF used for immunization was synthetized by PRIMM S.r.l. (Milan, Italy). A Cystein was added at the COOH-terminal to the ten amino acids long peptide (VSIPPRNLGY). The peptide was conjugated to Keyhole limpet hemocyanin (KLH) (VSIPPRNLGYC-KLH).

A 105 amino acids long fragment of human MSF (His-MSF; from residue 553 to 657 in the C-terminal portion of MSF, in red in Figure 8) including the specific MSF decapeptide at the C-terminus (in blue in Figure 8) and a histidine tag at the N-terminus (in black in Figure 8) has been produced in bacteria and purified by PRIMM. The recombinant fragment has a predicted molecular weight of 14.335 kDa.

```
        10          20          30          40          50          60
MGSDKIHHHH HHHHHHGVWK CDPVDQCQDS ETGTFYQIGD SWEKYVHGVR YQCYCYGRGI

        70          80          90         100         110         120
GEWHCQPLQT YPSSSGPVEV FITETPSQPN SHPIQWNAPQ PSHISKYILR WRPVSIPPRN

123
LGY
```

Figure 8. Sequence of His-MSF produced by PRIMM. The sequence of His-MSF expressed comprises the last 105 amino acids of human MSF (from residue 553 to residue 657, in red) and a 18 amino acid long tail containing 10 histidine (in black) at the NH2 terminus. The specific MSF peptide is indicated in blue and underlined.

3.2   Cell lines

CHO (Chinese hamster ovary), Hela (Human Cervical Cancer), A549 (Human Adenocarcinomic Alveolar Basal Epithelial cells), HEK293T (Human Embryonic Kindney), and MDA-MB231 (Human Breast Cancer) cells were cultured in Dulbecco Modified Eagle's Medium (DMEM) with 10% (v/v) Foetal calf serum (FCS). HCT 116, SW620, SW480, RKO, HT29 (Human Colon Carcinoma), 8387 (Human Fibrosarcoma), PANC1 and PT25 (Human Pancreatic Cancer) cell lines were cultured in RPMI with 10% FCS.

SP2/0 myeloma cells used for the generation of hybridomas were grown in DMEM with 10% FCS.

3.3 Animals

CD1 nude mice, CB17/SCID and C57Bl/6J mice were obtained from Charles River (Charles River Laboratories, Calco, Italy). Procedures involving animals and their care were conformed to institutional guidelines in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 86/609, OJ L 358,1,12-12-1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council 1996). The procedures used in the present study were approved by the Animal Care and Use Committee of the Istituto Clinico Humanitas and by Ministero della Salute (authorization No 09/2013-B).

Animals were housed in the specific pathogen free Animal Facilities at Istituto Clinico Humanitas. Groups of 5-7 mice were housed in individually ventilated cages with 12 hours dark/light cycle and *ad libitum* access to autoclaved food and water. A certified veterinarian was responsible for animal welfare supervision and regular health monitoring of the animal facilities. All efforts were made to minimize the number of animals used and their suffering. Animals were euthanized by cervical dislocation after sedation.

3.4 Gel electrophoresis and Western blot analysis

Polyacrylamide gels (10% w/v) were run in the discontinuous buffer system of Laemmli (Laemmli, 1970). Samples were diluted in 0.5M Tris-HCl pH6.8, 0.1% (v/v) Sodium Dodecyl Sulphate (SDS), 1% (v/v) Bromophenol blu, 3% (v/v) Glycerol, 10% (v/v) β-mercaptoethanol (sample buffer). Gels were stained with Silver Staining Kit (Sigma-Aldrich) following the manufacturer's instructions. For western blot analysis, proteins were transferred (1hour at 100 Volt) to nitrocellulose membrane (pore size 0.45μm, Biorad) using 1x Transfer Buffer TG [25 mM Tris, 192 mM Glycine pH 8.3, 20 % (v/v)

Methanol; Bio-rad]. To verify protein transfer after blotting, the membranes were stained with Ponceau S staining solution (Sigma-Aldrich). Membranes were then briefly washed with water and blocked for 1 h. at RT in blocking solution [5% (w/v) milk in PBS$^{+/+}$ containing 0.1% (v/v) Tween 20]. After blocking, filters were incubated overnight at 4°C with primary antibodies (RAB1 and RAB2 5μg/ml; mouse mAb α-His 3μg/ml) diluted in blocking solution. After three washes with washing buffer (PBS$^{+/+}$, 0.1% Tween 20), membranes were incubated with the appropriate anti-rabbit or anti mouse IgG-horseradish peroxidase conjugate, and finally developed with ECL (Enhanced Chemiluminescence, Immobilon Western, Millipore) according to the manufacturer's instructions. Precision Plus Protein Dual Color Standards (Bio-Rad, Milan, Italy) were used to determine the molecular weight of the proteins.

3.5 Protein evaluation

Total protein content was routinely evaluated by Bradford assay (Thermo Scientific, Milan, Italy). The concentrations of homogeneous preparations of recombinant MSF (i.e., as assessed by analytical SEC, SDS-PAGE and immunoblotting analyses) was determined by UV absorbance at 280 nm using a value of 150000 M^{-1} cm^{-1} for the protein's extinction coefficient [i.e., as computed by the ProtParam algorithm from the primary sequence of MSF (Gasteiger et al, 2003)].

3.6 Real-time PCR

Basal MSF gene expression was measured in different human cancer cell lines by Real Time-quantitative PCR (RT-qPCR) (Giulietti et al, 2001).Total RNA was extracted with PUREzol RNA Isolation Reagent (Biorad, Milan, Italy) as described above from MDA-MB-231, PANC1, HCT116, RKO, HT29, SW480, SV620, Hela, A549, A293T, 8387 cancer cell lines.

Forty ng of cDNA, 500 nM of forward and reverse primers (sequence are reported in Table 1) and 10 μl of SYBR MIX FAST (Applied Biosystem) in a final volume of 20 μl per reaction in 96-well format were used. Accumulation of fluorescent products was monitored using a 7900HT Fast Real-Time PCR System (Applied Biosystem). Specificity of PCR reaction was controlled by the melting temperature profiles of final products (dissociation curve). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as control housekeeping gene for normalization. Transcript fold enrichment was calculated by ΔΔct method (Livak & Schmittgen, 2001).

3.7 ELISA

3.7.1 Indirect ELISA

ELISA was used to analyse the specificity of polyclonal and monoclonal antibodies developed in this study. To this aim either the MSF specific peptide (VSIPPRNLGYC-KLH or recombinant His-MSF (section 3.1) were immobilized on plastic wells. In addition indirect ELISA was routinely used to detect human MSF in culture supernatant of transfected cells.

In general, ELISA plates (Nunc Maxisorb immunoplates) were coated (O/N at 4°C) with His-MSF (500 ng – 1 μg/well), the purified peptide (1 μg/well), human fibronectin1 (1 μg/well), or supernatant from transfected clones diluted in 15 mM Na carbonate buffer (pH9.6). Non-specific interactions were blocked by incubation for 2 h at room temperature with 5% (w/v) dry milk in PBS$^{+/+}$ and 0.05% Tween 20 (washing buffer). Wells were washed three times with washing buffer, then 10 -1 μg/ml of anti-MSF polyclonal antisera diluted in washing buffer were added and incubated 1 h at room temperature. Anti-rabbit IgG labelled with horse-radish peroxidase (GE Healthcare) were then added (1/2000 in washing buffer) and incubated 1 h at room temperature. Reaction was developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Thermo Scientific, Milan, Italy) and stopped with

2N H_2SO_4 before reading absorbance at 450 nm with an automated plate reader (Versamax microplate reader). Each sample was analysed in triplicate and results are reported as mean $OD_{450} \pm SD$ or SEM, as indicated in figure legends. Baseline was obtained adding anti-MSF antibody on PBS-coated wells.

3.7.2 Sandwich ELISA

To evaluate the amount of recombinant MSF produced by transfected cells we set up a sandwich ELISA. To this aim we tested different antibodies recognizing the N-terminal portion of FN1 conserved in human MSF as well as polyclonal antibodies and the monoclonal antibody MoAb_E10, recognizing MSF and developed in the present study (see sections 3.10 and 3.11). Acceptable results were obtained using the monoclonal antibody MAB1892, recognizing the Gel-BD domain of human FN1, as capturing antibody, while the polyclonal antibody RAB1.2 was used to specifically detect bound MSF. A standard curve was generated using a purified preparation of recombinant MSF (Lot # 3) previously quantified by UV absorbance. ELISA plates were coated (O/N at 4°C) with MAB1892 at a concentration of 1ug/ml in 15 mM Na carbonate buffer (pH9.6). Non-specific interactions were blocked by incubation for 2 h at room temperature with 5% dry milk in washing buffer. After washing, different amounts of MSF diluted in washing buffer were added (0.01-2.5 μg/ml) and incubated for 2 hours at 37°C. Then, wells were washed three times and incubated 1 h at room temperature with 1 μg/ml of RAB1.2 diluted in washing buffer. Anti-rabbit IgG labelled with horse-radish peroxidase (GE Healthcare) were then added (1/2000 in washing buffer) and incubated 1 h at room temperature. The plate was developed with TMB for 5 minutes and stopped with 1M H_2SO_4 before reading at 450 nm. The same general protocol was used to test MoAb_E10 as a possible capturing antibody in a sandwich ELISA (see the result section 4.1.3).

3.8 Generation of MSF expression vectors

The full length cDNA (2,192 bp, accession No. AJ535086.1) of human MSF subcloned into the BamHI site of pUC57 (2,710 bp) was obtained by GenScript.

The MSF cDNA insert was removed from pUC57 cloning vector (100 μg) by digestion with BamHI (60U) at 37°C overnight. The cDNA was purified from the agarose gel (Wizard SV Gel and PCR Clean-up System, Promega) and then ligated into pSG5 expression vector (4,100bp), previously linearized with BamHI, to obtain the pSG5-MSF expression vector. The orientation of the cloned MSF was initially analysed by digestion with XbaI, and then confirmed by sequencing.

One Shot® TOP10 Chemically Competent *E. coli* were transformed with pSG5-MSF in both the sense and anti-sense orientation and the plasmids were purified by PureYield™ Plasmid Midiprep System – (Promega). CHO cells were transfected using FuGENE Transfection Reagent (Roche) following manufacturer's instructions. Since the pSG5 expression vector does not contain any selection marker, cells were co-transfected with pSV2neo plasmid, which confers resistance to geneticin (G418). Transfected cells were cultured for two weeks in the presence of G418 (800 μg/ml) and then cloned by limiting dilution. Sense (CHO-cl1) and antisense (CHO-A3) clones were selected and used in this work. In addition CHO-cl1 was further subcloned by limiting dilution and CHO-3E6 clone, producing higher levels of MSF, was selected and used in some experiments (see section 4.1.2.2).

3.9 Purification of recombinant human MSF

To purify human MSF from the culture supernatant of transfected CHO cells we followed two different strategies of affinity chromatography based on the presence in MSF of an heparin-binding domain (Hep1/Fib1; Figure 8) or a gelatin-binding domain (Gel-BD).

a) Batch affinity purification on Heparin-sepharose beads CL-6B. 10 ml of supernatant from CHO-cl1 transfected cells were concentrated 10 times using a VIVASPIN column (Sartorius Italy, Muggiò, Italy), 30kDa MWCO (Molecular Weight Cut Off), and then dialyzed against Buffer E (0.1 M NaCl, 10 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.0). The concentrated supernatant was then incubated with 2ml of Heparin-sepharose beads CL-6B (GE-Healthcare), previously equilibrated overnight with Buffer E, at 4°C under rotation overnight. At the end of incubation the unbound material was removed by centrifugation and beads were washed 3 times with Buffer E and once with 20 mM sodium phosphate (1.64g Na_2HPO_4-7 H_2O, 0.47g NaH_2PO_4, pH 7.0 in 1L with H_2O). MSF (Lot # 1) was finally eluted using 0.5M NaCl - 10mM sodium-phosphate.

b) Affinity chromatography on Heparin-sepharose column followed by reverse phase HPLC. To obtain a greater amount of protein we purified 100 ml of supernatant from CHO-cl1 using a 20 ml column of Heparin-sepharose CL-6B. CHO-cl1 supernatant was diluted with equilibration/loading buffer (50mM Tris/HCl, 50mM NaCl, pH 7.4) before loading onto the column. The protein was eluted with a linear NaCl gradient (0.05-2 M NaCl in loading buffer (50mM Tris/HCl, 50mM NaCl, pH 7.4); 3ml-fractions were collected. All fractions were analysed by Western blot and indirect ELISA using rabbit antisera (see section 3.10). Fractions containing MSF were pooled and purified by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) using C-8 Aquapore RP-300 column (PerkinElmer, Norwalk, CT) or Source 5 RPC column; (Amersham Biosciences) equilibrated with 0.01% (v/v) trifluoroacetic acid (TFA) in H_2O. MSF (Lot # 2) was eluted with acetonitrile gradient (0-99.9% CH_3CN - 0.4 ml-fractions in 0.1% TFA).

c) Affinity chromatography on Gelatin-sepharose column followed by SEC. 150 ml of supernatants from CHO-cl1 were loaded on a 1ml Gelatin-sepharose 4B column. The column was equilibrated with $PBS^{-/-}$ at the flow rate 0.2 ml/min and then washed with 12

column volume of PBS$^{-/-}$ containing 1M NaCl followed by 50 mM NaAcetate, 200 mM NaBr, pH 5.0. Bound proteins were eluted with 50 mM NaAcetate, 1M NaBr pH 5.0, and concentrated with VIVASPIN 6 (10 kDa MWCO). The concentrated solution was then chromatographed on Superdex 200 10X300 Gel Filtration Column (GE Healthcare) equilibrated and eluted with PBS$^{-/-}$ at a flow rate of 0.5 ml/min (MSF Lot # 3).

d) Affinity chromatography on Gelatin-sepharose column followed by affinity chromatography on Heparin-sepharose column or SEC. For this purification we used CHO-3E6 cells derived from further subcloning of CHO-cl1. 400 ml of supernatants from CHO-3E6 were loaded on a 5 ml Gelatin-sepharose 4B column. The column was equilibrated with PBS$^{-/-}$ at the flow rate 0.5 ml/min. After this step the column was washed with PBS$^{-/-}$+ 1M NaCl and then with PBS$^{-/-}$. Bound MSF was eluted using a two steps gradient: 200 mM Arginine (10 ml of final volume), and 1M Arginine pH 5.22 (24 ml final volume). Eluted MSF was dialyzed against 50mM Tris-HCl, 50mM NaCl pH 7.4 (40 ml final volume).

To separate MSF from contaminating FN1, 30 ml of eluted MSF were loaded on a Heparin-sepharose CL-6B column and eluted using a three step gradient of elution in three steps. Briefly, 50 mM Tris was used with the addition of three different concentrations of NaCl (0.5M, 1M and 2M). The eluted preparation (MSF Lot # 4) was dialyzed against PBS$^{-/-}$ and loaded onto Superdex 200 10X300 Gel Filtration Column (0.5 ml/min).

In parallel, 10 ml of the eluted preparation from Gelatin-sepharose column were concentrated 20 times by VIVASPIN 30.000 (30 kDa MWCO) and directly loaded onto a Superdex 200 10X300 Gel Filtration Column with flow rate 0.5 ml/min (MSF lot # 5).

3.10 Generation of polyclonal antibodies

Rabbit polyclonal antisera were generated by PRIMM immunizing rabbits with the synthetic peptide specific for human MSF detailed in section 3.1. Pre-immune serum was collected (1ml/rabbit), then rabbits were challenged intraperitoneally (ip) with 300 µg of the MSF-specific peptide conjugated to KLH (VSIPPRNLGYC) diluted in Complete Freund Adjuvant (CFA). Immunization was repeated at day 21, 28 and 35 with the peptide diluted in Incomplete Freund Adjuvant (IFA). Blood (40-50 ml/rabbit) from immunized rabbits was collected and tested by ELISA before the purification of specific antibodies. Polyclonal antibodies (pAbs) anti-MSF were purified by immunoaffinity on a CNBr-Sepharose column carrying the peptide.

3.11 Generation of monoclonal antibodies (mAb) against MSF

PRIMM was also involved in the generation of monoclonal antibodies against human MSF. Mice were immunized with a four-branched multiple antigenic peptide (MAP) (Figure 9) in which four copies of the MSF-specific peptide were conjugated through the α- and ϵ- amino functional groups of lysine to an inert core. The result is a large macromolecule that does not require further conjugation to a carrier protein. Immunization and first evaluation of antibody titers by ELISA were performed by PRIMM, while fusion and screening of hybridomas was conducted in our laboratory.
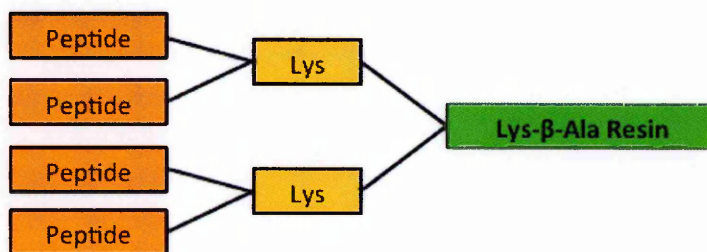
Figure 9. Structure of MAP-peptide. The MAP (Multiple Antigenic Peptide) system utilizes the α- and ϵ- amino functional groups of lysine to form an inert core to which four chains of MSF-specific peptide are anchored.

Based on ELISA the evaluation of antibody titers in the immunized animal, the spleen from one mice was collected and sent to our laboratory. Mouse splenocytes, obtained after dissociating spleens with a syringe needle, were fused with SP2/0 myeloma cells (5:1 ratio) using polyethylene glycol 1550 (SERVA, Rome, Italy) (Manufacturer's standards procedures) Cells were seeded in 96 well plates and selected using HAT medium (RPMI-1640 medium containing 10% FCS, 100 mg/mL streptomycin, 100 IU/mL penicillin, 100 mM hypoxanthine, 16 mM thymidine, and 400 mM aminopterin). After 2 weeks, culture supernatants of surviving clones were screened for antibody reactivity and specificity by indirect ELISA on His-MSF. The screening was repeated twice and 5 out of 23 positive clones (6H3, 3D3, 4G4, 5H4, 6B10) were selected and subcloned two times by limiting dilution. The hybridomas were analysed by indirect ELISA against His-MSF, supernatant from CHO-3E6 cells and FN1. This allowed us to select 9 hybridomas recognizing recombinant MSF but not FN1.

3.12 Purification of monoclonal antibodies by protein G column

$3-5 \times 10^6$ cells from selected hybridomas were injected in the peritoneal cavity of CD1 nude mice after pre-treatment 1 week before with 1 ml of Pristane (Sigma-Aldrich).

Ascites (approximately 5ml) were collected 10 days after injection of hybridomas and MSF-specific antibodies were purified by Protein G-sepharose 4 Fast Flow column (Pharmacia, Milan, Italy) by standard procedures. Briefly, protein G-sepharose columns (1 ml column volume) were equilibrated with PBS$^{+/+}$ and loaded with 1 ml of the different ascites diluted 1:3 in PBS. The flow-through was applied again onto the column and the procedure was repeated 3 times. MSF-specific antibodies were then eluted using 0.1 M Glycine-HCl pH 2.8 and immediately buffered using 1.5 M Tris-HCl pH8.8.

The isotype of the different monoclonal antibodies was determined using the Mouse Monoclonal Antibody Isotyping Test Kit MMT1 (Biorad).

3.13 MSF expression in polarized macrophages

Human monocytes were isolated from buffy coats obtained from normal blood donors by Ficoll (Hystopaque-1077; Sigma-Aldrich) and Percoll (GE-Healtcare) gradient centrifugation as previously described (Solinas et al, 2010). Residual T and B cells were removed from monocytes by plastic adherence.

To obtain tumour-conditioned macrophages (TC-MΦ), 3×10^6/ml monocytes were cultivated for 5 days in RPMI 1640 containing 5% FCS in the presence of 30% (v/v) of supernatant derived from human pancreatic carcinoma cell lines PT45 or PANC1 (Solinas et al, 2010). Cells were incubated for additional 24 h with LPS (100 ng/ml) and IFN-γ (25 ng/ml) or IL-4 (20 ng/ml; all from PeproTech) to obtain M1- or M2-polarized MΦ, respectively. Total RNA extraction from MΦ was performed with TRIzol (Invitrogen). following manufacturer's instructions. cDNA was synthesized using 1 μg of total RNA and the GeneAmp RNA PCR kit (Applied Biosystems) according to the manufacturer's instructions. 40 ng of cDNA were used as template in a total reaction volume of 25 μl containing 12.5 μl Taq Polymerase RedMix (Bioline) and 0.5 μl of each primer (10 μM). The list of primers used to analyse MSF mRNA expression by PCR is reported in Table 1. The reactions were analysed on 1% (w/v) agarose gel.

3.14 Migration assay

Migration of human pancreatic tumour cells (PANC1) was evaluated using a transwell system with 8 μm pore size (6.5mm - Costar, Euroclone, Milan, Italy). For the migration assay cells were harvested by brief exposure to trypsin, extensively washed and resuspended in DMEM without FCS. Purified MSF was also diluted in DMEM without serum and added to the lower chambers of the 24-well format transwells, while 1% FCS in DMEM was used as positive control. Cancer cells (1×10^5) were added to the upper chamber of the transwell and incubated for 6-24 h at 37°C. At the end of the incubation, transwells were recovered and non-migrated cells were removed from the top of the

membranes using cotton swabs. Filters were then fixed in methanol, and stained with Diff-Quick (Medion Diagnostics). Migrated cells were counted in 20 high-power oil-immersion fields (HPF). Each sample was analysed in triplicate and results are expressed as the mean number of migrated cells in 20 HPF ± SD.

The same protocol was used to investigate the migration of other cancer cell lines, namely a breast cancer cell line, MDA-MB231; some colon cancer cell lines, HT29, SW480 and SW620; and CHO-3E6 cells.

Migration of peripheral blood monocytes was evaluated using a chemotaxis microchamber technique as previously described (Locati et al, 2001). Briefly, 27 µl of control medium (RPMI 1640) or purified MSF were added to the lower wells of a chemotaxis chamber (Neuroprobe, Pleasanton, CA, U.S.A.). The cell suspension (50 µl of a solution containing 1.5×10^6 monocytes/ml) was seeded in the upper chamber. The two compartments were separated by a PVP polycarbonate filter (5 mm pore size; Neuroprobe). The chamber was incubated at 37°C in humidified atmosphere in the presence of 5% CO_2 for 90 min. In these experimental conditions, using a PBMC suspension only monocytes migrate across the filter and are recovered on its lower side. At the end of incubation, filters were removed, stained with Diff Quick, and 5 HPF were counted. Each sample was analysed in triplicate and results are reported as mean migrated cells ± SD.

Migration of murine peritoneal exudate cells (PECs), mostly macrophages, was analysed essentially with the same protocol. Briefly, PECs were obtained from C56Bl/6J female mice 24 h after intraperitoneal injection of PBS^{-/-} and adjusted to a concentration of 2×10^6 macrophages/ml. Five µl of this cell suspension were plated in the upper compartments of the microchamber. After 4 h at 37°C filters were removed and processed as described above for human monocytes.

3.15 *In vivo* growth of MSF transfected cells

In vivo growth of cells expressing MSF (CHO-cl1 or CHO-3E6) and CHO-A3 was analysed in CB17/SCID female mice. A range of different amounts of cells (from 3×10^6 to 5×10^4; as detailed in section 4.2.3) in 200ul of $PBS^{-/-}$ were injected into the left limb of mice. Tumour appearance was followed daily and tumours were measured by a calliper three times a week. Tumour volume was calculated following the formula: $(dm^2 \times DM)/2$ (dm: minor diameter; DM: major diameter). Some tumours were collected at different time from injection and fixed in OCT (Optimal Cutting Temperature compound) for immunohistochemical analysis.

3.16 Immunohistochemical analysis

MSF expression was evaluated in OCT-embedded tumors or in paraffin-embedded specimens of tumours from mice injected with MSF-expressing CHO-cells (5 μm-thick histologic sections) and in paraffin-embedded human tissue sections (3 μm-thick histologic sections) from lung, breast and colon cancer.

Immunohistochemical analysis of mouse tumour sections

Sections from OCT-embedded tumor specimens were fixed in 4% (v/v) paraformaldehyde (PFA) for 15 minutes at room temperature (RT). Sections were washed with 0.05% Tween 20 in $PBS^{-/-}$ for 5 minutes and incubated with Peroxidase 1 (Biocare Medical, Space Import Export, Milan, Italy) for 5 minutes followed by incubation with Roden Block (Biocare Medical) for 30 minutes.

Sections were stained with primary antibodies diluted in Da Vinci Solution (Biocare Medical) for 1 hour at RT. As primary antibodies we used the rabbit polyclonal antiserum RAB 2.3 (5 μg/ml), and the murine monoclonal antibody MoAb_E10 (1.5 μg/ml) generated in our laboratory. After the incubation with primary antibodies the different sections were incubated (30 minutes at RT) with the appropriate HRP-conjugated secondary antibody, namely the anti-rabbit MACH-4 Universal HRP-Polymer (Biocare

Medical) for 30 minutes, or the Mouse-on-Mouse HRP-Polymer (Biocare Medical) for 30 minutes, respectively. Color was developed using 3,3'-diaminobenzidine (DAB; BioCare Medical) free base as chromogen, and then the sections were counterstained with hematoxylin (Dako).

To identify macrophages, paraffin-embedded tissue samples were stained with F4/80 (AbD Serotec). Before staining, sections were dewaxed in Xilene or Bioclear, with 2-3 washes for 10 minutes each. After dewaxing, sections were hydrated with a rapid wash in ethanol (2 washes), ethanol 90% (v/v), ethanol 70% and then in water. Antigen unmasking was performed in a Decloaker Chamber in DIVA Buffer 1X (Biocare Medical) for 3 minutes at 125°C and 5 minutes at 90°C. Endogenous peroxidases were blocked with Peroxidized-1 (Biocare Medical) for 20 minutes or using 3% (v/v) hydrogen peroxide for 20 minutes. After washing with PBS-/-, 0.05% Tween 20, non-specific binding sites were blocked with Rodent Block M solution (Biocare Medical) for 30 minutes. Sections were then incubated with monoclonal rat anti mouse F4/80 at 1 µg/ml overnight. Rat on mouse HRP polymer kit (for F4/80) was finally used to detect F4/80 staining. The reactions were developed with DAB. Slides were counterstained for 2 min with haematoxylin and mounted with mounting medium Eukitt.

Immunohistochemical analysis of on human tumour sections

Paraffin-embedded human tissues sections were cut and kept overnight at 37°C. Sections were dewaxed in Xilen or Bioclear, as detailed above. Antigen unmasking was performed in a Decloaker Chamber in DIVA Buffer 1X (Biocare Medical) for 3 minutes at 125°C and 5 minutes at 90°C. After blocking endogenous peroxidases with Peroxidized-1 or with 3% (v/v) hydrogen peroxide for 20 minutes, non-specific binding sites were blocked with Background Sniper solution (Biocare Medical) for 30 minutes. Human tissue samples were then stained with MoAb_E10 (2.5 µg/ml for 1h) to identify MSF, or with mouse monoclonal anti-human CD68 (Dako; 780 µg/ml for 1h) to identify macrophages.

Staining with CD68 does not require antigen unmasking. Immunostaining with MoAb_E10 or with CD68 was revealed following incubation with rabbit anti-mouse MACH1 Polymer-HRP (Biocare Medical) for 15 minutes. Then the reactions were developed with 3,3'-Diaminobenzide (DAB). Slides were counterstained for 2 min with haematoxylin and mounted with mounting medium Eukitt.

3.17 *In situ* hybridization

Sense and antisense mRNA probes for human MSF were designed using Primer3 (Rozen & Skaletsky, 2000) and the sequences are reported in Table 2. The nucleotides in green indicate the T7 sequence (for the forward primer) and T3 sequence (for the reverse primer). The PCR product is 151 bp long.

Antisense and sense riboprobes labelled with ^{35}S-UTP were generated by *in vitro* transcription with T3 and T7 RNA polymerases (Riboprobe Combination System T3/T7- Promega), following Manufacturer's instructions. The template was digested with DNase I (Riboprobe Combination System T3/T7- Promega), and non-incorporated ^{35}S-UTP and DNA were removed by chromatography using Sephadex G50 columns (Amersham- GE Healtcare).

Table 2. Primers used for *in situ* hybridization		
Primers	Sequence of Primers (5'→3')	bp[a)]
Forward	5'-TAATACGACTCACTATAGGTATCCCACCCAGAA ACCTTG-3'	151
Reverse	5'-AATTAACCCTCACTAAAGGTTGACCACATATTG TTTGTTCACT-3'	

[a)] Amplicon size

The activity of each probe was adjusted to 1×10^7 cpm/ml using hybridization buffer (50mM DTT, 10% (w/v) Dextran Sulfate, 50% (v/v) formamide, 0.3M NaCl, 10mM Tris-HCl pH 7.6, 10mM NaH$_2$PO$_4$ pH 6.8, 5mM EDTA pH 8, 0.2% (w/v) Ficoll 400, 0.2% (w/v) polivinilpirrolidone). Paraffin-embedded sections were dewaxed in xylene and

rehydrated in graded ethanol. Proteins were removed first by acid treatment, incubating sections in 0.2M HCl for 15 min at RT, and then by incubating with 40 μg/ml proteinase K from Tritirachium album (Sigma-Aldrich) in 50 mM Tris-HCl pH7.6, 5 mM EDTA pH8.0, for 7 minutes at RT. Sections were then postfixed with 4% (w/v) PFA-PBS for 15 minutes at RT. Subsequently all sections were acetylated in order to neutralize positive charges, eliminating non-specific signals. Sections were then air-dried at RT before labelling with ^{35}S-labeled probes (1x10^6 cpm/slide in hybridization solution) incubating overnight at 50° C in a humidified chamber.

After hybridization, sections were washed 2 times (45 minutes at 65°C) with FS solution (2X SSC, 50% (v/v) formamide) and then 2 times (30 minutes at 37°C) with STE solution (2X SSC, 20 mM Tris-HCl pH7.6, 1mM EDTA pH8.0). Sections were subsequently treated with RNase A (10 μg/ml in 0.5 M NaCl, 1 mm EDTA, 10 mm Tris-HCl, pH 7.2) for 30 minutes at 37° C to remove nonspecifically bound riboprobes. Finally sections were washed with STE solution for 10 minutes at 37°C and then 2 times with FS solution for 45 minutes at 65°C, before air-drying at RT. At this point sections were exposed to high performance autoradiography film (Amersham Hyperfilm – GE Healthcare) for 3 days, then dipped in Kodak photo emulsion NTB-2 for 3 weeks. Development and fixation were performed according to the Kodak protocol, and the sections were counterstained with hematoxylin (Dako) before evaluation with the dark field condenser U-DCD (Olympus BX51).

3.18 Statistical analysis

Statistical analysis was performed using Graphpad Prism (Prism 4 for Windows, version 4.03). Data were compared by unpaired two tailed Student's *t* test or by one way Anova followed by Dunnett's post-test. P values of < 0.05 were considered statistically significant. Linear regression analysis was used to construct a standard curve for the setting of a sandwich ELISA.

4 RESULTS

The selective expression of MSF by M2-like polarized TAM suggested that this molecule could play a role in the tumour microenvironment (Solinas et al, 2009). This prompted us to analyse MSF in the context to tumour biology. Given that no commercial reagents (i.e. recombinant MSF or anti-MSF antibodies) were available, considerable efforts have been devoted to the characterization of reagents, either developed in-house or outsourced, useful for the investigation of the role of MSF in human tumours. Thus the Results section is divided into three parts, the first concerning the production and characterization of reagents, the second part describing the biological studies performed with recombinant MSF, and the last part concerning the evaluation of MSF expression in mouse and human tumours.

4.1 Characterization and production of new reagents for human MSF

4.1.1   Generation of rabbit polyclonal antibodies against MSF

Different lots of pAbs were developed by PRIMM immunizing rabbits with the specific MSF peptide (see section 3.10) and were used in the present study, i.e RAB1, RAB2, RAB1.2, RAB2.2, RAB1.3 and RAB2.3. Each lot was characterized by ELISA against the immobilized peptide (100 ng/well). Figure 10a reports a typical titration curve of RAB1 and RAB2. The rabbit pAbs obtained do not recognize human fibronectin 1 (FN1) while they can recognize a recombinant fragment of human MSF (His-MSF; see below) produced by PRIMM (Figure 10b).
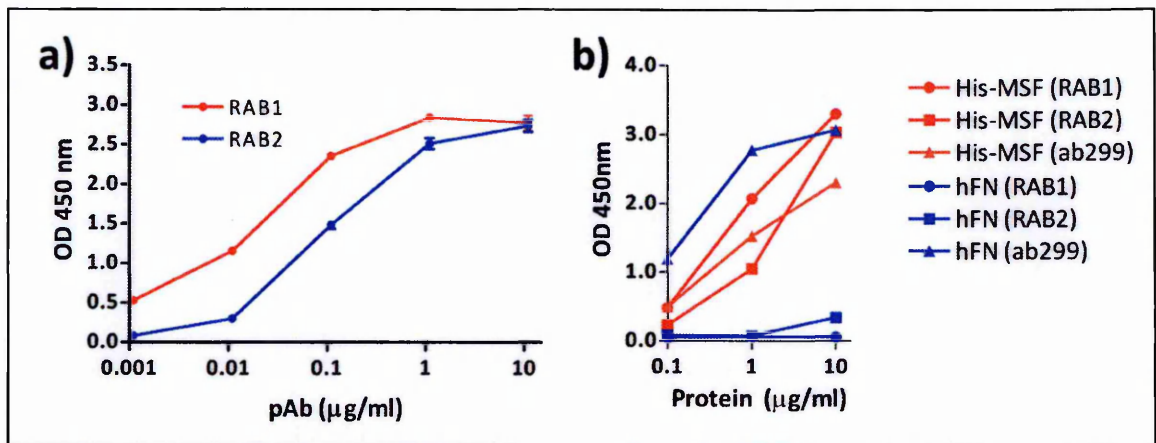
Figure 10. Specificity of anti-MSF pAbs. Specificity and sensitivity of rabbit pAbs were analysed by ELISA. a) ELISA plates were coated with 100 ng/well of the synthetic peptide VSIPPRNLGY. Different concentrations of the two rabbit pAbs RAB1 and RAB2 were then added. Results are reported as mean $OD_{450}\pm SD$ of triplicate wells for each point. b) Specificity of pAbs anti-MSF was verified by ELISA. Different concentrations of His-MSF and FN1 were immobilized on plastic wells and incubated with RAB1 or RAB2, used at 10µg/ml, or with a rabbit polyclonal antibody against human FN1 (ab299) used at 1µg/ml. Results are reported as mean $OD_{450}\pm SD$ of triplicate wells.

Similar results were obtained with RAB1.2, RAB2.2, RAB1.3 and RAB2.3. (Figure 11a and b)
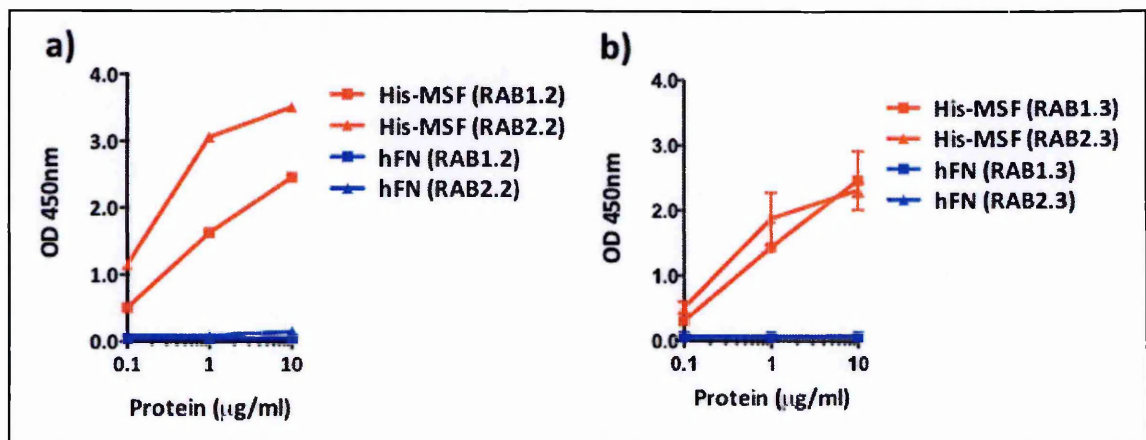
Figure 11. Specificity of different lots of anti-MSF pAbs. Specificity of pAbs anti-MSF was verified by ELISA. Different concentrations of His-MSF and FN1 were immobilized on plastic wells and incubated with RAB1.2 and RAB 2.2, RAB 1.3 or RAB2.3 (all used at 10 µg/ml). Results are reported as mean $OD_{450}\pm SD$ of triplicate wells.

4.1.2 Expression and purification of recombinant protein

4.1.2.1 Production of a recombinant fragment of MSF protein (His-MSF)

The His-MSF fragment produced by PRIMM (see section 3.1) is recognized in ELISA (Figure 10b; Figure 11a and 11b) and western blotting (Figure 12b) by the pAbs generated in the present study. As shown in the Figure 12a and b, both silver staining and western blot analysis identify the recombinant fragment His-MSF with an apparent molecular weight of 14 kDa, as expected. In addition, a upper band is evident, with an apparent molecular weight of 28 kDa, that likely represents a dimer of His-MSF due to the cross-link between the cysteines included in its sequence.
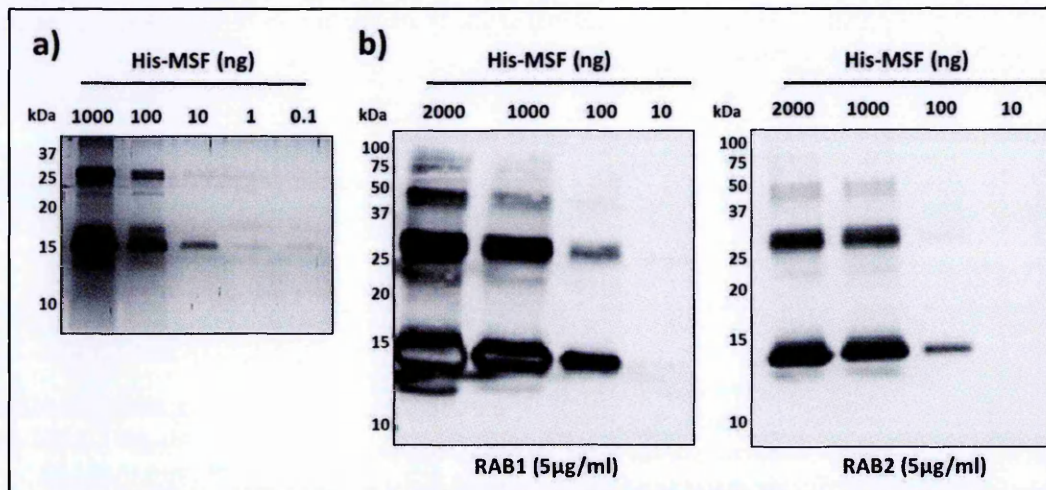
Figure 12. Analysis of the recombinant fragment His-MSF. Recombinant human His-MSF was characterized by SDS-PAGE followed by silver staining or western blotting. a) Different quantities of His-MSF (from 1000 to 0.1ng of total protein) were separated on 10% polyacrylamide gel and then silver stained. b) different quantities of His-MSF (from 2000 to 10 ng of total protein) were separated on 10% polyacrylamide gel and transferred to membranes for western blotting. Analysis was performed with RAB1 or RAB2 antibodies (both at 5µg/ml), as indicated.

4.1.2.2 Expression of human recombinant MSF protein

The MSF cDNA insert (2,192bp) coding for human MSF was removed from pUC57 vector by digestion with BamHI and was sub-cloned in pSG5 vector (4,100bp), previously linearized with BamHI, in order to obtain the pSG5-MSF expression vector. The orientation of the cloned MSF was initially analysed by digestion with XbaI (Figure 13a),

and then confirmed by sequencing for two selected constructs identified to have insert in the sense or anti-sense orientations (Figure 13b). As shown in Figure 13c, after digestion with XbaI the sense orientation is characterized by two fragments, respectively of 1,617 bp and 4,675 bp, while the antisense orientation shows two fragments of 895 bp and 5,397 bp
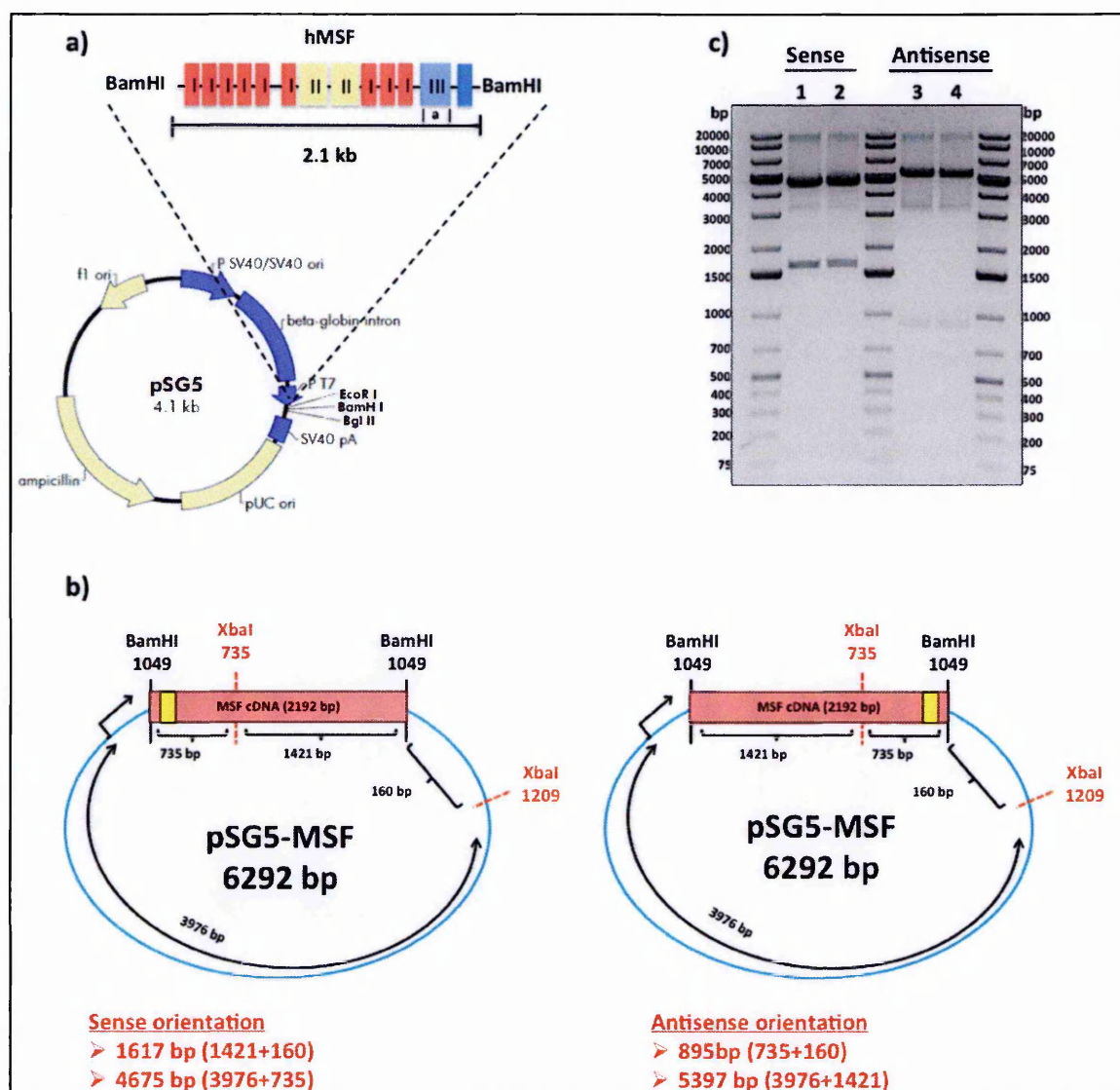
Figure 13. MSF expression vector. a) Schematic representation of pSG5-MSF expression vector: the 2.1 kb fragment of MSF has been cloned in the BamHI site of pSG5 (4.1 kb); b) schematic representation of pSG5-MSF containing the insert in sense and antisense orientation. c) to verify the orientation different clones of pSG5-MSF were digested with XbaI and aliquots were separated on 1% agarose gel. Lanes 1 and 2: pSG5-MSF with the insert in the sense orientation; lane 3 and 4: pSG5-MSF with the insert in the antisense orientation. Expected length of the fragments obtained after digestion with XbaI are reported in Panel b.

To obtain recombinant human MSF, CHO cells were co-transfected with pSG5-MSF and pSV2-neo, that confers resistance to G418 (see section 3.8). Supernatants from over

300 clones were analyzed by dot blot analysis. His-MSF (1 μg) was used for comparison. As evident in figure 14a, most of the clones did not produce appreciable levels of MSF, however we identified 24 clones showing MSF production. Given that MSF content in the supernatant of transfected clones was heterogeneous, we selected 6 clones with an apparent high MSF expression for further analysis. First we analyzed the supernatants by SDS-PAGE followed by western blot in order to verify the apparent molecular weight of the protein produced by transfected clones. Since at this stage we did not know the sensitivity of the polyclonal antibodies RAB1 and RAB2 for the full length MSF protein, incubation was performed with both the antibodies. Results reported in Figure 14b indicated that the most abundant protein has an apparent molecular weight of approximately 75 kDa, as expected on the basis of MSF protein sequence. In parallel two clones transfected with the anti-sense expression vector were analysed and clone CHO-A3 was selected for further experiments.
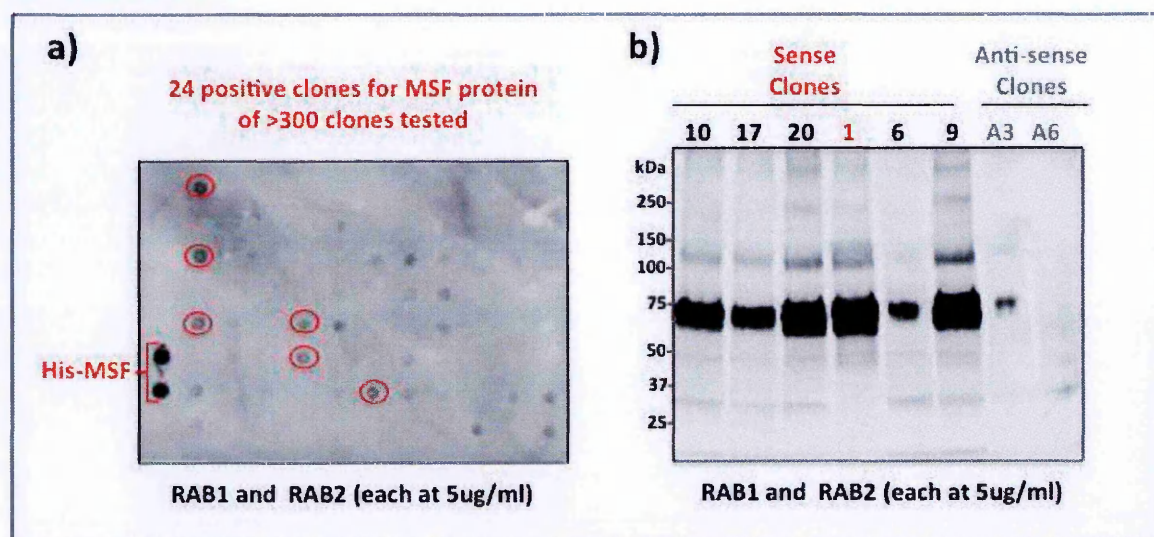

Figure 14. Expression of human MSF protein. a) Dot blot analysis of supernatants from clones obtained by limiting dilution of transfected CHO-cells. Red circles identify some of the MSF positive clones. His-MSF (1μg) was used as a positive control. b) Western blot analysis of six clones producing MSF and two clones transfected with the anti-sense construct. MSF protein is identified using RAB1 and RAB2 polyclonal antiserum (each at 5μg/ml).

Clones 1, 9 and 20 showed the highest signal in western blot, thus supernatants from these clones were further analysed by indirect ELISA (Figure 15). To this aim the same cell number of clones 1, 9 and 20 were plated and supernatants were collected after 24 hrs.

Figure 15 reports the results obtained with RAB1 and similar data were obtained when RAB2 was used (not shown). As evident in Figure 15 supernatants from the three sense clones (CHO-cl1, CHO-cl9, CHO-cl20) but not from the antisense clone, contain MSF. In order to optimize the selection of a cloned cell population, CHO-cl1 was further sub-cloned by limiting dilution. Two clones were identified, CHO-3E6 and CHO-3G4, both secreting MSF in the culture supernatant (Figure 16).
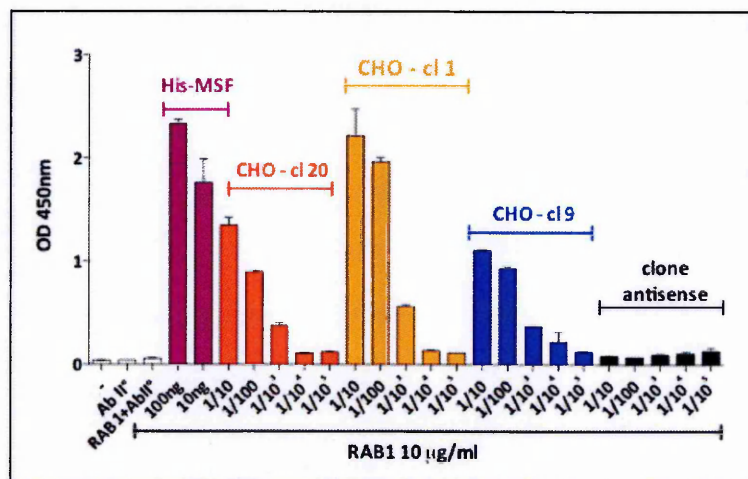
Figure 15. ELISA on supernatants from transfected clones. His-MSF (10-100 ng) or the indicated dilutions of supernatants from three different positive clone (CHO-cl20, CHO-cl1 and CHO-cl9) and from CHO-A3 (anti-sense clone) were coated on plastic wells. Presence of MSF in the culture supernatants was revealed by incubation with RAB1 (10μg/ml). Results are reported as mean $OD_{450} \pm SD$ of triplicate wells.
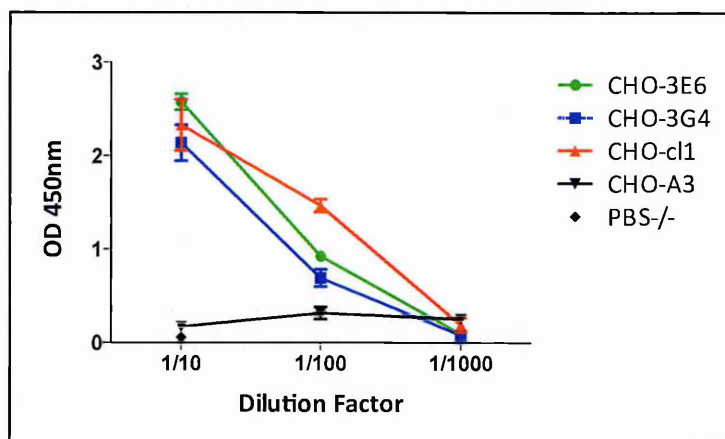

Figure 16. ELISA on supernatants from selected transfected clones. Supernatants from CHO-cl1, two derived subclones, CHO-3E6 and CHO-3G4, and antisense clone CHO-A3, were coated on plastic wells and the polyclonal antibody RAB2.3 (1μg/ml) was used to identify MSF. Results are reported as mean $OD_{450} \pm SD$ of triplicate wells. OD_{450} obtained when RAB2.3 was incubated in PBS-coated wells is reported as negative control.

4.1.2.3   Purification of MSF protein

MSF was purified from the supernatant of transfected cells taking advantage of the presence of two different domains: the heparin binding domain and the gelatin-binding (Gel-BD) domain (Figure 7). In a preliminary experiment we tested the feasibility of a purification process using Heparin-sepharose beads. Ten ml of concentrated supernatant from CHO-cl1 were incubated with Heparin-Sepharose beads and bound proteins were eluted with 0.5M NaCl, 10mM sodium phosphate (pH 7.0). Eluted proteins were then separated by SDS-PAGE and analysed by silver staining and western blot (Figure 17a and b). As shown in Figure 17b, a band with an apparent molecular weight of 75 kDa recognized by anti MSF pAbs is evident in lanes 5 and 6, where we loaded the fractions eluted from Heparin-Sepharose beads. This indicates that we were in fact able to partially purify human MSF. However, silver staining (Figure 17a) also showed that a series of contaminants were still present in fractions 5 and 6, suggesting that the protocol needed to be improved. In particular a band of approximately 50 kDa, present in the supernatant from CHO-cl1 cells (lane 1) is still evident in lanes 5 and 6, where eluted MSF has been loaded. Thus we took advantage of a long standing collaboration with Jo Van Damme and Sofie Struyf at the Rega Institute in Leuven, two experts in the field of chemokine purification, and with their help we set up a two-step purification protocol based on affinity chromatography on Heparin-Sepharose followed by reverse phase HPLC. Silver staining and western blot analysis (Figure 18a and b) of fractions 23 through 30 eluted after HPLC, showed a defined 75 kDa band correspondent to human MSF (lot 2), but again some contaminants with different molecular weights were still present (Figure 18a).
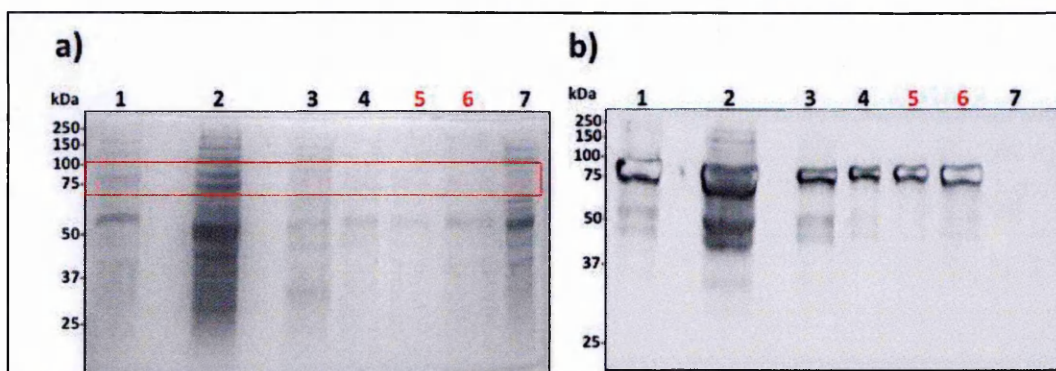
Figure 17. Purification by affinity to heparin-sepharose. Supernatant from CHO-cl1 cells was concentrated 10 times and incubated with Heparin-Sepharose beads in a Eppendorf tube. After removal of the unbound material and washing with 1 ml of Buffer E, MSF was recovered by incubation with 0.5M NaCl - 10 mM sodium-phosphate (pH7.0). Samples were loaded on 10% polyacrylamide gels. Two gels were run in parallel, one was silver stained (a) and the other was transferred to a membrane for western blotting (b). RAB1+RAB2, both at 5 μg/ml were used to identify human MSF. Lanes are as follow: 1) supernatant from CHO-cl1 cells (20µl); 2) concentrated supernatant (40 µl); 3) Unbound material (40 µl); 4) washing with Buffer E (40 µl); 5) and 6) eluted MSF (40 µl); 7) supernatant from CHO-A3 cells (40 µl) .
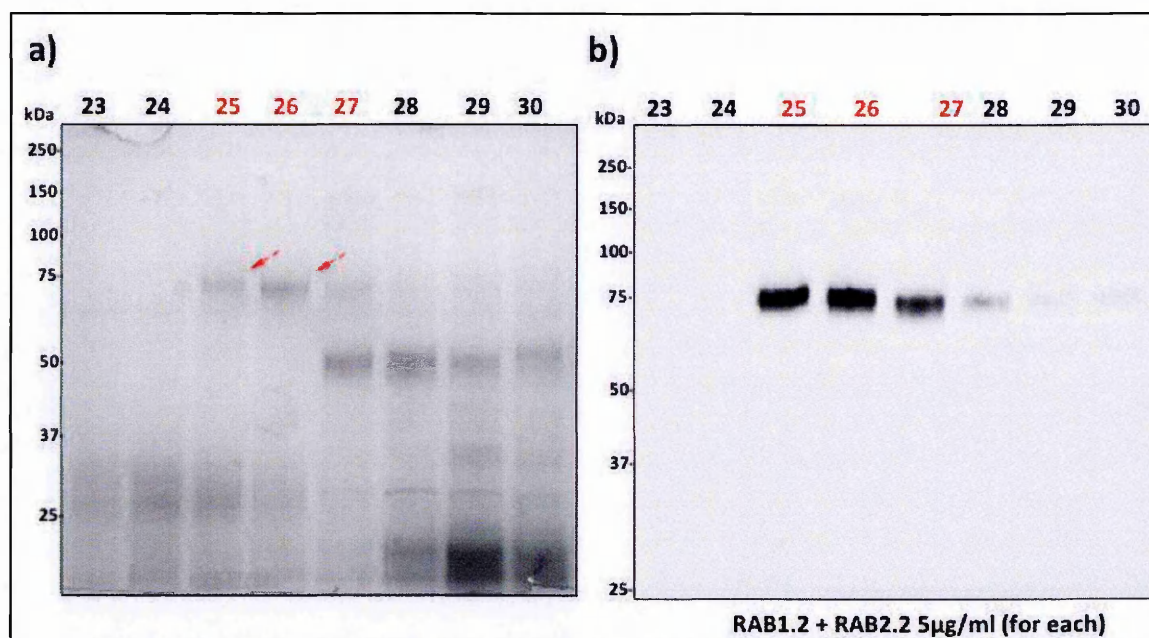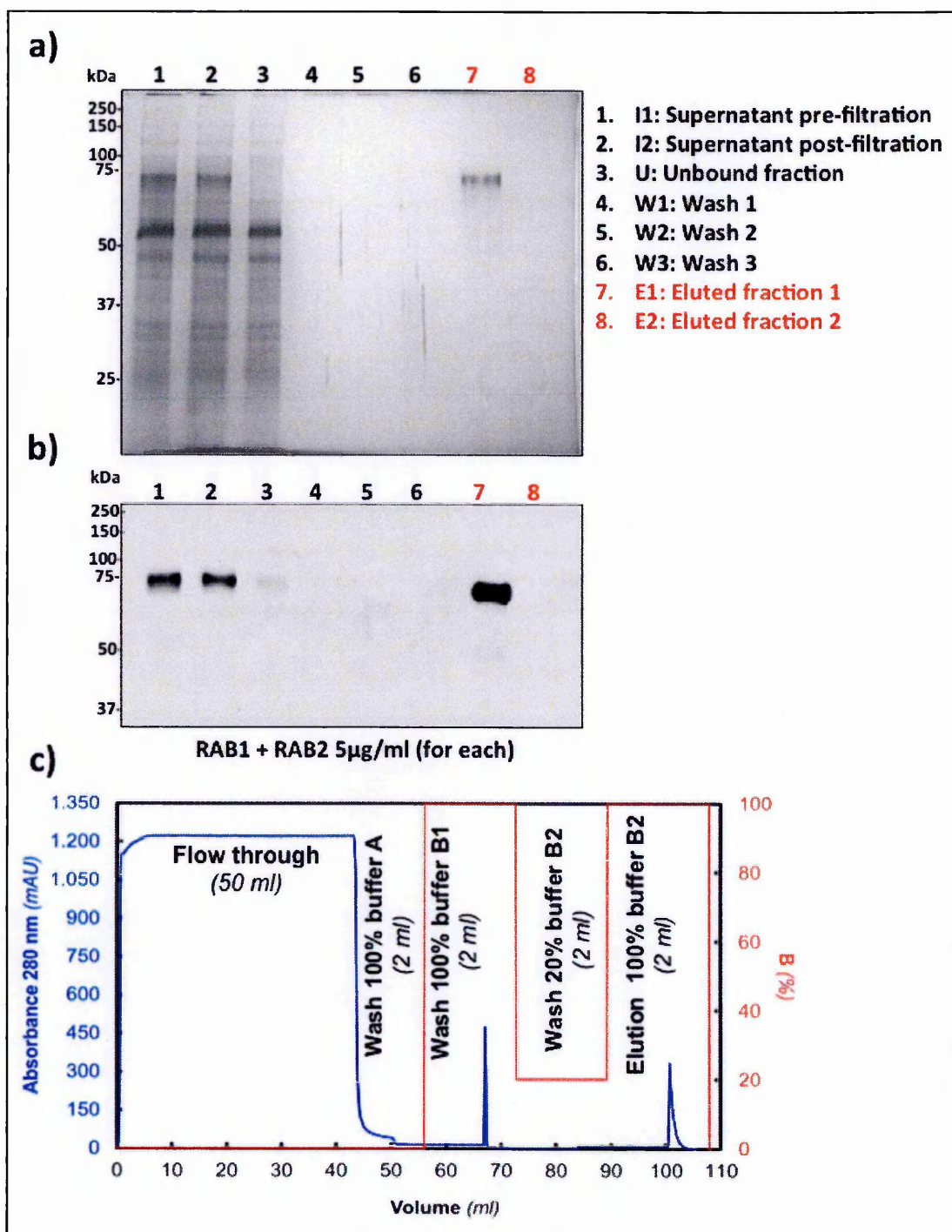

Figure 18. Purification by Heparin-affinity chromatography and HPLC. Supernatant from CHO-cl1 was first separated on a Heparin-sepharose column. The eluted fractions containing MSF were further subjected to HPLC. Fractions 23 through 30 were eluted by HPLC and analysed by 10% SDS-PAGE. a) Silver staining of HPLC fractions. Red arrows indicate MSF (Lot 2). b) Western blot analysis of HPLC fractions performed with RAB1.2+RAB2.2 polyclonal antisera (both at 5 μg/ml).

To further improve the purification process, we tested an alternative strategy taking advantage of the presence in MSF of the high affinity Gel-BD domain. Therefore, supernatant from CHO-cl1 cells was purified by affinity chromatography on a Gelatin-Sepharose column followed by Size Exclusion Chromatography (SEC). Given that FN1

also contains the Gel-BD and the heparin binding domains, this protein could represent a possible contaminant of our preparations. Thus we introduced SEC to separate the proteins considering their molecular weight, approximately 250 for FN1 and 75 for MSF. Silver staining performed on the different fractions of the purification process (Figure 19a) showed a sharp and relatively pure band at the expected molecular weight (75 kDa) in lane 7 (lot 3), and western blot analysis confirmed that the band obtained was indeed human MSF (Figure 19b). Chromatogram of the purification process through Gelatin-Sepharose column is reported in Figure 19c.

A last strategy for MSF purification was based on a first step of affinity chromatography on Gelatin-sepharose 4B column followed either by affinity on Heparin-sepharose (Lot 4) or by SEC (Lot 5). As detailed in Section 3.9, 400 ml of supernatant from CHO-3E6 were loaded on the Gelatin-sepharose column. The column was washed first with 1M NaCl and then with PBS-/-. MSF was eluted with a two-step gradient of Arginine: the first step at 200 mM was not sufficient to recover the protein while the second step at 1 M Arginine allowed the detachment of MSF from Gelatin sepharose. The eluted MSF was dialyzed as detailed in section 3.9 and then loaded on Heparin-sepharose or SEC columns. Aliquots of the different steps of the purification processes were analysed on 10% SDS-PAGE in two parallel gels, one was silver stained (Figure 20a) and the other was transferred to a membrane for western blotting (Figure 20b). The silver staining performed on the different steps of the two purification processes showed a slight fibronectin contaminant in the fraction eluted from Heparin-sepharose column (Figure 20a – lane 9). On the contrary, purification by SEC resulted in an almost pure preparation of MSF, as shown by the single band at the expected molecular weight observed by SDS-PAGE (Figure 20a – lane 10). Western blot with RAB1 and RAB2 pAbs is reported in Figure 20b.

Figure 19. Purification by Gelatin-affinity chromatography and SEC. Supernatant from CHO-cl1 cells was purified by affinity chromatography on Gelatin-Sepharose column. After removal of the unbound material, MSF was eluted with 50 mM Na-acetate, 1M NaBr. Samples of the different steps were analysed by 10% SDS-PAGE. a) Silver staining b) western blot performed with RAB1+RAB2 polyclonal antisera (both at 5 μg/ml). c) chromatogram of the purification through Gelatin column.
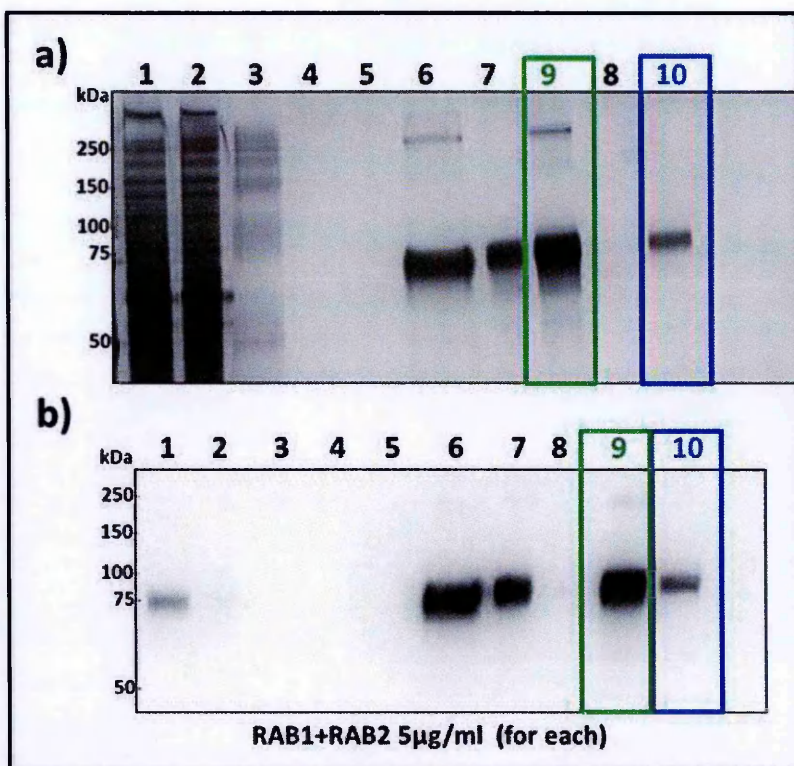
Figure 20. Purification by affinity chromatography on Gelatin-sepharose 4B column followed either by affinity chromatography on Heparin-sepharose CL-6B column or by SEC. Aliquots of the different steps of the two purification processes were loaded on 10% SDS-PAGE. a) Silver staining b) western blot performed with RAB1+RAB2 pAbs (both at 5 µg/ml). Lanes are as follow: 1) input Gelatin-sepharose column (supernatant from CHO-3E6); 2) unbound material (Gelatin-sepharose column); 3 & 4) washing respectively with 1M NaCl and PBS-/-; 5) 1st step with Arginine (200mM); 6) 2nd step with Arginine (1M); 7) input Heparin or SEC column (protein eluted with 1 M Arginine and dialyzed); 8) unbound material (Heparin-sepharose column); 9) MSF eluted from Heparin-sepharose column; 10) MSF eluted from SEC.

In summary, the different lots of purified MSF are reported in Table 3.

| Table 3. Lots of recombinant human MSF obtained with the different strategies of purification				
Lot No	Source of Supernatant	1° STEP of Purification	2° step of Purification	Recovery
Lot 1	CHO-cl1	Affinity purification on Heparin-sepharose beads CL-6B		118 µg/ml (118 µg tot.)
Lot 2	CHO-cl1	Affinity chromatography on Heparin-sepharose beads CL-6B	RP-HPLC (Reverse-Phase High Performance Liquid Chromatography)	n.d.*
Lot 3	CHO-cl1	Affinity chromatography on Gelatin-sepharose 4B column	SEC (Size Exclusion Chromatography)	47 µg/ml (23 µg tot.)
Lot 4	CHO-3E6	Affinity chromatography on Gelatin-sepharose 4B column	Affinity chromatography on Heparin-sepharose beads CL-6B	100 µg/ml (100µg tot.)
Lot 5	CHO-3E6	Affinity chromatography on Gelatin-sepharose 4B column	SEC (Size Exclusion chromatography)	10 µg/ml (35 µg tot.)

*n.d : not determined

4.1.3 Generation and purification of mouse monoclonal antibodies against MSF

No monoclonal antibodies against human MSF are commercially available, thus the generation of such reagents has been fundamental to the success of the project.

Two different mouse monoclonal antibodies are described: a mouse monoclonal antibody (mab7.1) was raised against the MSF-specific decapeptide. The epitope recognized by mab7.1 was identified as PPRNLGY by pepscan analysis (Schor et al, 2003). A second mouse monoclonal antibody described (1D2 antibody) was generated by Hu H. and colleagues (Hu et al, 2009). In this case the monoclonal antibody reacted strongly with cell surface antigens of human esophageal cancer endothelial cells (HECEC) and was selected because could influence their migratory behaviour (Hu et al, 2009).

Given the high identity between human MSF and FN1, and between human and murine FN1, the generation of monoclonal antibodies represented a big challenge. For this reason we outsourced part of the work to PRIMM, who defined and produced the immunogens, performed immunizations and evaluated the response of mice to the challenge; instead, the screening of hybridomas was performed in our laboratory. Animals were immunized with a branched version of the decapeptide (MAP-peptide) synthetized by PRIMM (see section 3.11). Antibody titers were evaluated initially by PRIMM and subsequently in our laboratory. Data reported in Figure 21 refers to a titration experiment by ELISA performed in our laboratory and reports the data obtained with sera from two animals with results essentially similar to those obtained by PRIMM (not shown).
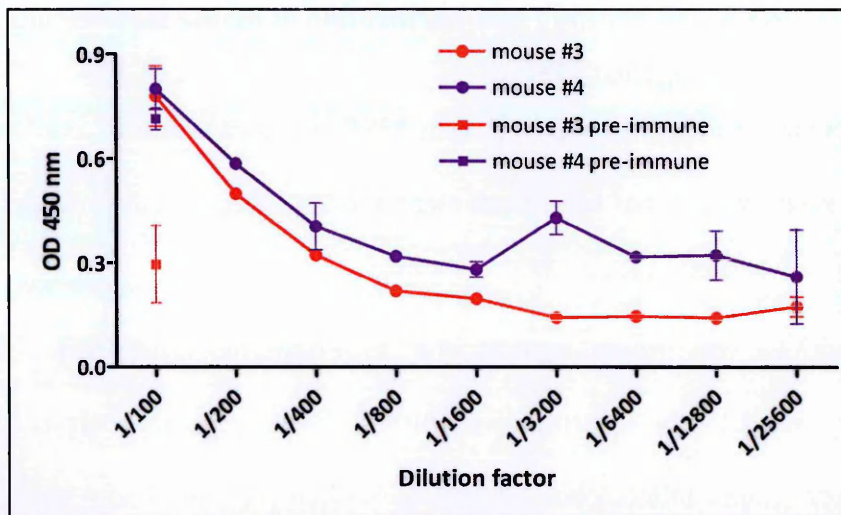
Figure 21. Screening of sera from mice immunized with MAP-peptide. ELISA with of sera obtained from Balb/c mice immunized with MAP-peptide. His-MSF (1µg/well) was immobilized onto plastic wells and different dilutions of sera from immunized mice were added. Each point represents mean $OD_{450} \pm SD$ of triplicate wells.

Splenocytes were obtained from an immunized mouse with a satisfactory titer and fused with SP2/0 myeloma. The first screening of the fusion was conducted by ELISA on immobilized His-MSF. In total we analysed supernatants from 616 wells. To select the hybridomas producing anti-MSF antibodies we classified the clones comparing OD_{450} to PBS, as indicated in Table 4.

| Table 4. Production of anti-MSF antibodies from hybridomas.									
Response[1]		**No wells / Plate**							**Percentage on total wells[3]**
		1	**2**	**3**	**4**	**5**	**6**	**7**	
Absent	Baseline[2]	87	85	86	84	83	86	87	97.1
Low	Twice baseline	1	2		3	2	1	1	1.6
Medium	Three to five time baseline			1		1	1		0.5
High	> five time baseline		1	1	1	2			0.8

[1] Response was classified comparing OD_{450} obtained with supernatants from hybridomas and baseline.
[2] Baseline refers to PBS used instead of supernatant from hybridomas (range OD_{450} 0.15 - 0.5).
[3] Total wells analyzed in seven plates were 616.

As shown in Table 4, most of the hybridomas did not produce anti-MSF antibodies, while five hybridomas showed a high response in terms of OD_{450}. This analysis was repeated a second time for 27 selected hybridomas representative of the different levels of

response, and results of the two ELISA are reported in Figure 22. Among the twenty seven hybridomas, the five showing a high response in the two ELISA assay (3D3, 4G4, 5H4, 6B10, 6H3) were sub-cloned at 5 cells/well. Growing clones were tested again in ELISA. Five clones were selected following the criteria detailed above, and subcloned at 1 cell/well. From this second subcloning, we obtained in total almost 118 clones that were tested in ELISA on His-MSF, recombinant MSF and FN1. Twelve hybridomas, recognizing His-MSF and recombinant MSF, but not FN1, were finally selected (Figure 23). To increase the amount of antibody production, we injected hybridomas into CD-1 nude mice. To this purpose we to injected nine of the hybridomas reported in Figure 23. After 1 week, ascites fluids were recovered and tested by indirect ELISA on His-MSF (Figure 24).
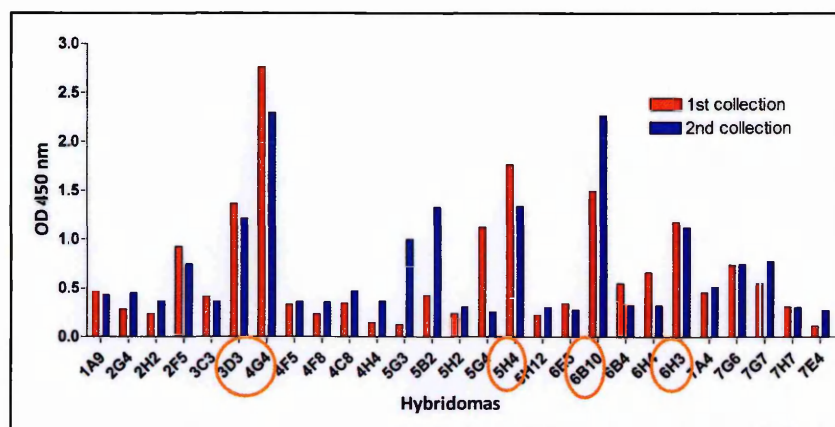

Figure 22.Screening of supernatants from 27 selected hybridomas. Supernatants from hybridomas were analyzed by indirect ELISA on wells coated with His-MSF (1μg/well). The experiment was repeated twice with supernatants collected in two different days to confirm reproducibility of the results. Five positive clones (circle) were selected and further subcloned .
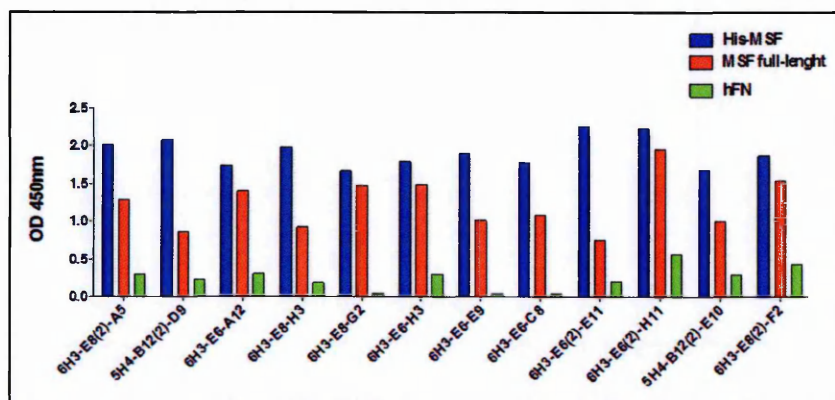

Figure 23. Final screening of monoclonal antibodies. Clones were analysed by indirect ELISA against His-MSF, MSF full-length and human FN1 immobilized onto plastic wells (1μg/well).
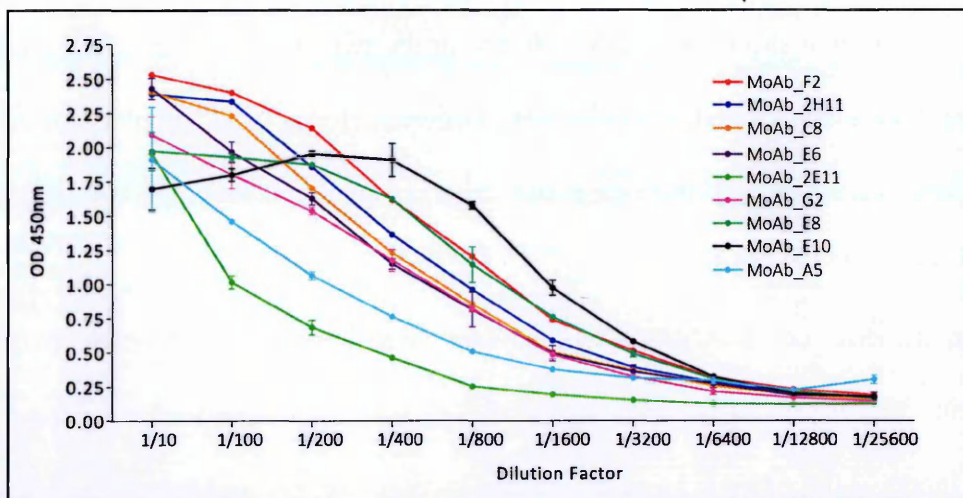
Figure 24. Titration of ascites fluids. Ascites fluids were titrated by indirect ELISA on His-MSF (1μg/well) immobilized on plastic wells. Different dilutions of the ascites collected from mice were tested. Results are expressed as mean OD_{450} ±SD of triplicate wells.

A first group of antibodies in ascites fluids were purified by protein G sepharose. Figure 25 reports the titration curve by direct ELISA of MoAb_E10, MoAb_C8 and MoAb_F2. Coating was performed with different dilutions of supernatant from CHO-3E6 cells, while purified antibodies were all used at 1 μg/ml. MoAb_E10 resulted to have the best titer while MoAb_F2 and MoAb_C8 showed a lower efficiency in the recognition of recombinant MSF. Thus MoAb_E10 was selected for further experiments. Analysis of the isotype indicated that MoAb-E10 is an IgM.
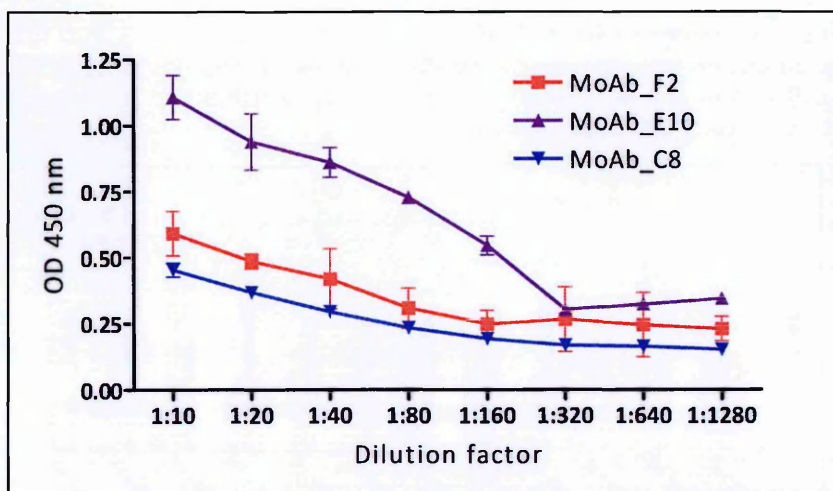
Figure 25. Titration of purified antibodies on full-length MSF. Purified MoAb_E10, MoAb_C8, MoAb_F2 (1μg/ml) were plated on wells coated with different dilutions of supernatant from CHO-3E6 cells. Each sample was analysed in triplicate and data are expressed as mean OD_{450}± SD.

### 4.1.4 Development of a sandwich ELISA to quantify MSF in biological fluids

In order to quantify MSF in biological fluids we tried to optimize a sandwich ELISA specific for the protein. Among the different combinations of antibodies tested, optimal results were obtained with two capturing antibodies: a commercial monoclonal antibody recognizing the Gel-BD domain of human FN1, that is also conserved in MSF (MAB1892), and the murine MoAb_E10 developed in our laboratory. The rabbit antiserum RAB1.2, that does not recognize FN1, was used to detect captured MSF. Recombinant MSF purified by affinity chromatography on Gelatin-Sepharose followed by SEC (Lot 3) was used as standard (0.02-2.5 µg/ml). Figure 26 shows the standard curve obtained when MAB1892 was used to capture MSF and RAB1.2 to detect bound MSF.
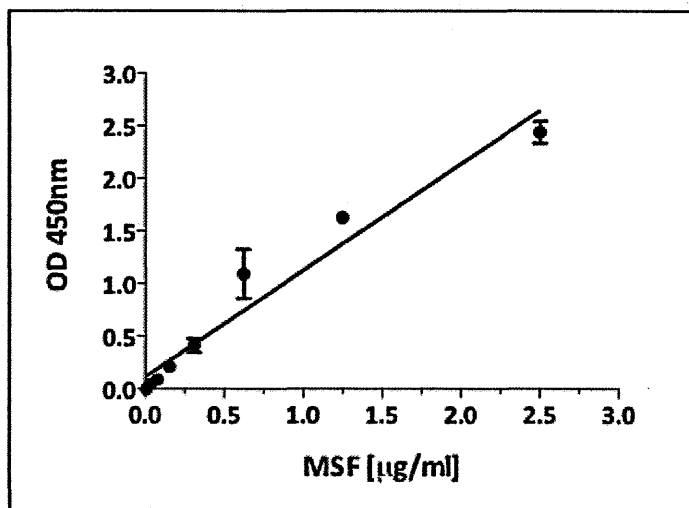
Figure 26. Standard curve with purified MSF. Plates were coated with MAB1892 (100 ng/well) and different concentrations of MSF (Lot 3) were used to generate a standard curve. RAB1.2 was used to detect bound MSF. Range of linearity was between 0.02 and 2.5 µg/ml (r^2= 0.9528; p<0.01).

The assay developed has a sensitivity of approximately 30 ng/ml. With this set up, we were able to detect MSF in the supernatants of transfected CHO cells, while there was no signal detected when supernatants of anti-sense transfected CHO cells (CHO-A3) were used as control (Figure 27a). This experiment confirmed that FN1, that is also present in the culture supernatants of CHO cells and is captured by MAB1892, is not recognized in this setting (Figure 27a). Contrary to this, FN1 was detected in both the supernatants from CHO-cl1 and CHO-A3 when the rabbit polyclonal antibody ab299, recognizing human

FN1, was used to detect bound proteins (Figure 27b). In this case also FN1 captured by MAB1892 is detected, as expected, by ab299. As baseline, wells coated with MAB1892 were incubated with PBS and then with RAB1.2 or ab299. One way Anova with Dunnett's multiple comparison test was used to compare baseline OD_{450} obtained with PBS with those obtained with supernatants from CHO-cl1 and CHO-A3 cells or FN1 (* p<0.05; ** p<0.01).
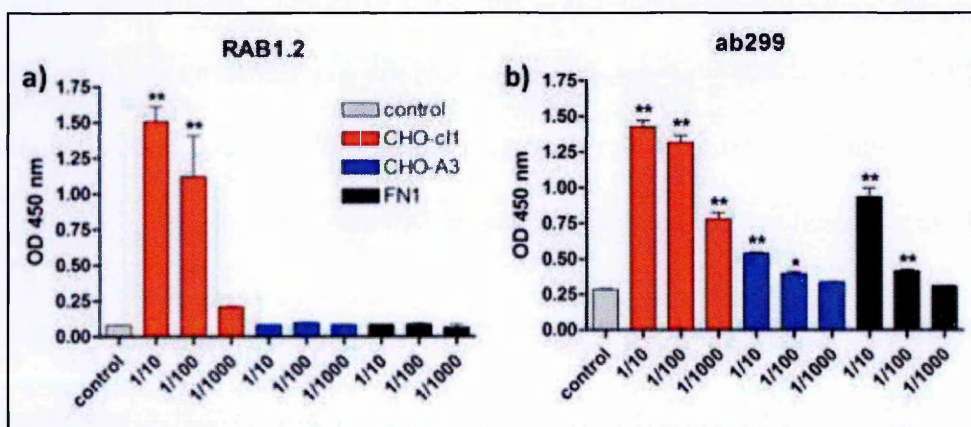

Figure 27. Detection of MSF in the supernatants of transfected CHO-cells by sandwich ELISA. Wells were coated with MAB1892 and then incubated with various dilutions of culture supernatants from CHO-cl1 and CHO-A3 (control) cells and human purified FN1. Detection of bound proteins was performed with a) RAB1.2 (anti human MSF) or b) ab299 (anti human FN1). Baseline is represented by wells coated with MAB1892 and then incubated with the appropriate detection antibody. Results are expressed as mean OD_{450} nm ± SD of triplicate wells. * p<0.05; ** p<0.01, (one way Anova with Dunnett's multiple comparison test) compared to PBS.

Using this experimental set up we made an initial estimation of the amount of MSF produced by CHO-cl1, which resulted to be approximately 8 μg/ml, and by CHO-3E6, that resulted to be approximately 40 μg/ml. This assay was also used to quantify MSF protein in biological fluids (plasma *vs* serum) of healthy donors. Initial experiments indicated that MSF can be detected in 5 out 7 plasma samples (Table 5). In this small group of subjects analysed, two of them had undetectable levels of MSF, four of them had a level in the range between 20 and 51 ng/ml, and one plasma sample had approximately a ten times higher amount of MSF (221 ng/ml). In addition this assay was not able to measure MSF in serum, with the exception of one subject (Table 5).

Table 5. MSF levels in plasma and serum of healthy subjects measured by sandwich ELISA		
Subject No	Plasma (ng/ml)	Serum (ng/ml)
1	22.9	ND*
2	ND	ND
3	221.1	43.5
4	50.9	ND
5	28.1	ND
6	25.1	ND
7	ND	ND

*not detectable

MoAb_E10 was also investigated as the capturing antibody in the sandwich ELISA. His-MSF and purified recombinant MSF (Lot 4) were used for the standard curve and bound proteins were detected with RAB1.2. With this setting we were able to detect His-MSF (Figure 28) with a sensitivity of approximately 40 ng/ml however the detection of purified MSF was poor and the lower limit of detection was 1.2 µg/ml (data not shown).
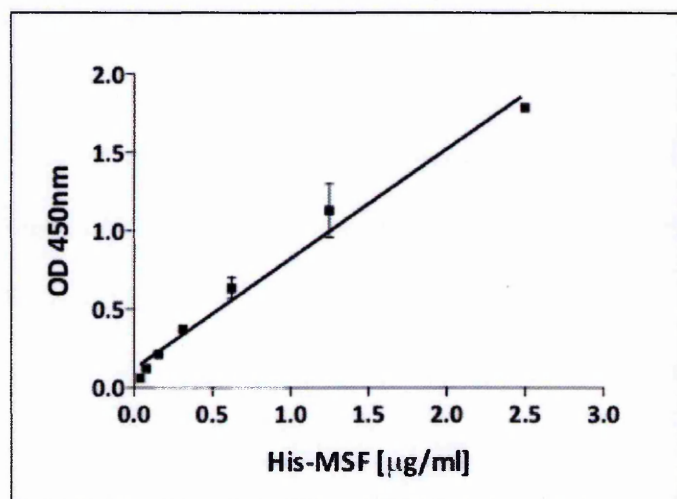
Figure 28. Standard curve with His-MSF. Plates were coated with MoAb_E10 (10 µg/ml) and different concentrations of His-MSF were used to generate a standard curve. RAB1.2 was used to detect bound MSF. The range of linearity was between 0.04 and 2.5 µg/ml ($r2= 0.9822$; $p<0.01$).

In summary we have synthetized and purified recombinant MSF and developed a monoclonal antibody (MoAb_E10) that recognizes specifically MSF. In addition we were able to set up a preliminary assay for the measurement of MSF in biological fluids. The reagents developed are suitable for the characterization of some of the biological properties of MSF and the monoclonal antibodies obtained can be tested in immunohistochemistry to

investigate MSF expression in human tumours. Sections 4.2 and 4.3 will describe the results obtained with these reagents.

4.2 Biological studies on human MSF

Once having available transfected cells expressing human MSF, purified recombinant MSF and monoclonal /polyclonal antibodies recognizing human MSF, we addressed more deeply the possible involvement of MSF in cancer. In particular we focused our attention on the motogenic properties of MSF and on the possible pro-tumoral role of this protein.

4.2.1 MSF expression in macrophages and cancer cells.

Solinas *et al.* (2010) found by transcriptional profiling that human MSF is expressed by M2-polarized macrophages. To confirm this finding, we induced macrophage differentiation from peripheral blood monocytes using conditioned medium from tumour cells (TC-MΦ). Macrophages were further polarized toward M1 or M2 phenotype by exposure to LPS and IFN-γ or IL-4 respectively. To evaluate MSF expression, total RNA was extracted and analysed by PCR using two sets of primers: CL1, amplifying a sequence present in the 5' end of both MSF and FN1, and CL2, amplifying a sequence specific for the 3' end of MSF (see section 3.1 and Table 1). As shown in Figure 29, the 1448 bp product amplified by CL2 primers is present only in TC-MΦ and in M2-polarized macrophages but not in M1 cells. On the contrary, the 771 bp fragment amplified by CL1 primers is present in all samples analysed. Therefore, while all monocytes and macrophages express FN1, only M2 and TC-MΦ express MSF, the former at an apparent higher level.
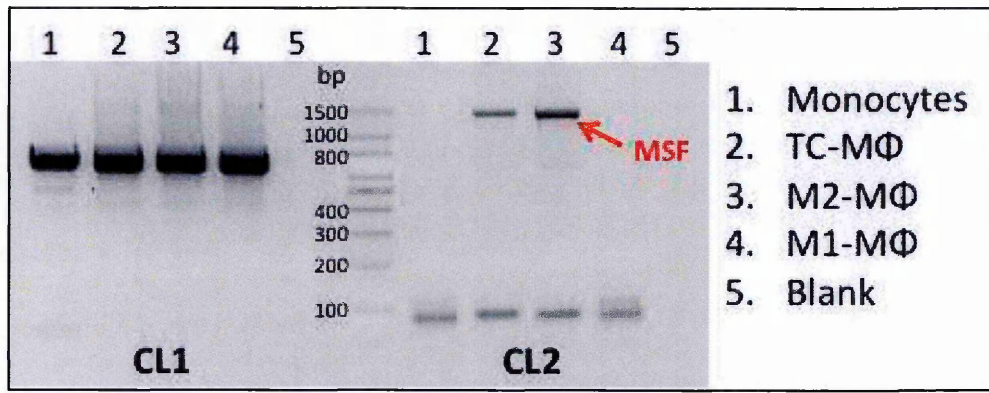
Figure 29. MSF expression in TC-MΦ. Agarose gel electrophoresis of amplified DNA from monocytes, TC-MΦ and polarized macrophages.

In addition, since MSF is also produced by tumour cells (Schor & Schor, 2010), expression was measured by RT-qPCR in human cancer cell lines of different origin (Figure 30). Here it was found that MSF was highly expressed in 8387 fibrosarcoma and in MDA-MB231 breast carcinoma, whereas other human cancer cell lines showed very low (or no) expression. These results suggest that MSF might be expressed in a tissue (and tumour) specific manner. The finding of MSF expression in M2 and M2-like macrophages as well as in tumour cells, might suggest a role in the regulation of tumour maintenance.
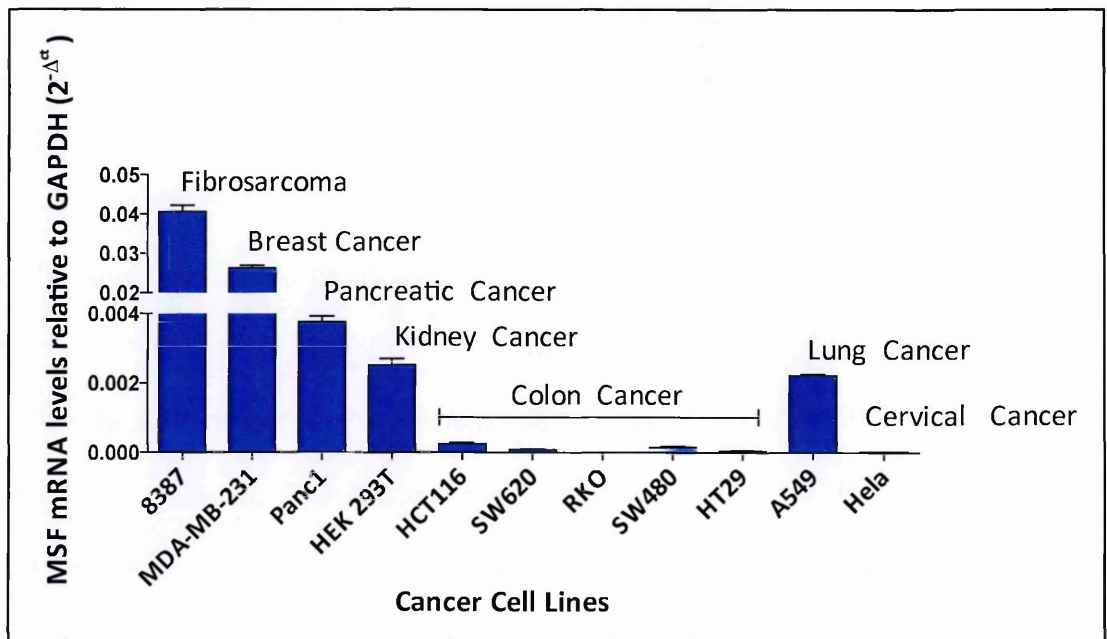
Figure 30. MSF expression in human cancer cell lines. RT-qPCR was used to quantify MSF expression in the indicated human cancer cell lines. Results are normalized to GAPDH expression. Each sample was analysed in triplicate and data are expressed as mean $2^{-\Delta ct} \pm$ SEM.
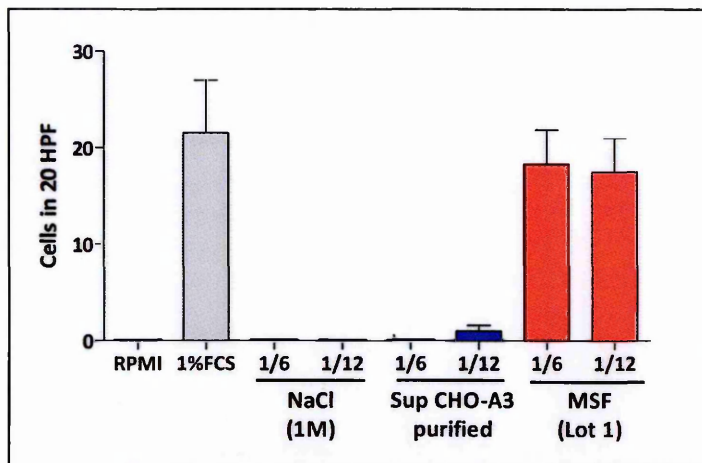
4.2.2 Motogenic activity of human MSF

Having available a small amount of recombinant MSF, we investigated its biological properties focusing initially on the motogenic activity. The transwell migration assay was used to evaluate the motogenic activity of MSF on Panc1 tumour cells, as described by Solinas *et al.* (2010) A first set of experiments was performed to set up the optimal conditions for Panc1 migration. Different incubation times (6 and 24 hours) and different concentrations of FCS (1% and 10%) as positive control for the assay were used. As can be seen in Figure 31, 1% FCS had the best effect in the induction of Panc1 migration. Given the higher number of migrated cells after 24 h incubation at 37°C, we opted for a 6 h incubation time.
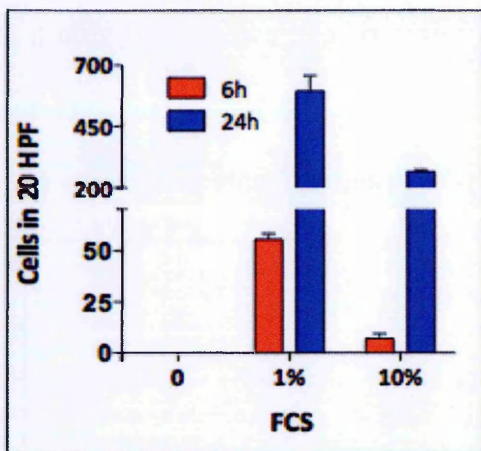
Figure 31. Migration of Panc1 cells. Migration of Panc1 cells was analysed using transwell plates. 1% and 10% FCS were used as a positive control. 10^5 Panc1 cells were allowed to migrate for 6 and 24 h at 37°C. Results are expressed as mean number ± SEM of cells migrated in 20 HPF.

Once the optimal setting was defined, we tested the effect of partially purified MSF (Lot 1) on migration of Panc1 cells. Lot 1 was used at a dilution of 1/6 or 1/12; as control supernatant from CHO-A3 was treated in the same way and the fraction eluted from Heparin-sepharose with 1M NaCl was tested at a 1/6 and 1/12 dilutions. As shown in Figure 32, MSF Lot 1 induces the migration of Panc1 cells to levels comparable to the migration observed with 1% FCS used as positive control. In the same setting, no motogenic effect is evident when similar dilutions of a comparable fraction from CHO-A3 cells or NaCl were tested. These results confirmed the motogenic activity of our purified MSF protein.

Figure 32. Motogenic activity of human MSF. Panc1 cells (10^5) were placed in the upper compartment of a transwell and incubated 6h at 37°C. Supernatant from CHO-A3 cells purified through Heparin-sepharose and NaCl (originally 1M) used for the elution of Heparin-sepharose column were tested as negative control at the same dilution used for MSF Lot 1. 1% FCS was used as positive control. Data are reported as mean number ± SEM, of migrated cells in 20 HPF. Each condition was tested in triplicate

We then moved to analyse the migration of monocytes toward purified MSF. Monocytes from healthy donors were allowed to migrate in a Boyden microchamber to different concentrations of purified MSF (1 – 100 pg/ml correspondent to 0.013 nM-1.3 nM considering a molecular weight of 75kDa) for 90 minutes at 37°C. Both preparations, purified respectively by affinity chromatography on Gelatin sepharose and then Heparin-Sepharose (Lot 4) or SEC (Lot 5) were used. Figure 33a shows the results obtained with MSF Lot 4: migration was observed with 10 pg/ml of MSF. The number of migrated monocytes was only slightly lower than the response to the supernatant from 8387 cells, a known source of MCP1/CCL2 used as positive control for monocyte migration.
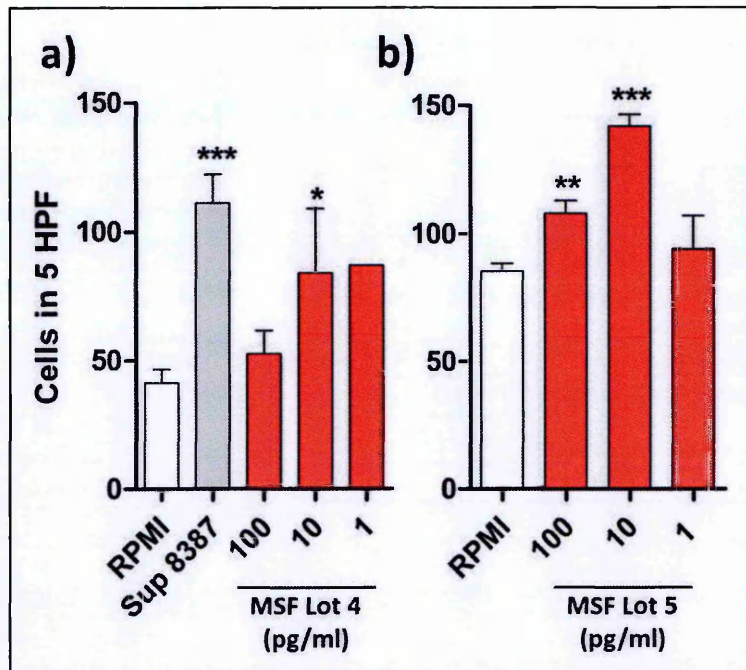
Figure 33. Migration of monocytes to purified human MSF. Monocytes were plated in the upper compartment of a Boyden microchamber while stimuli were added in the lower compartments. Negative and positive controls were respectively RPMI w/o FCS and supernatant from 8387 fibrosarcoma cells as source of CCL2/MCP1. a) MSF lot 4 and b) MSF lot 5 were tested at 100, 10 and 1 pg/ml. Each sample was analysed in triplicate or duplicate and data are expressed as mean number of migrated cells in 20 HPF $\pm$ SEM. Student's t test was used to compare migration to MSF or Sup 8387 and baseline migration to RPMI w/o FCS. * $p<0.05$; ** $p<0.01$; *** $p<0.0001$ compared to RPMI in the respective experiment.

When MSF lot 5 was used similar results were obtained, with a significant migration to 100 and 10 pg/ml of purified MSF (Figure 33b).

We also analysed the effect of endogenous MSF on cell motility. Initially we analysed the migratory behaviour of transfected CHO-3E6 cells compared to wild type CHO cells and we observed that the transfected CHO-3E6 cells, producing recombinant MSF, migrated more efficiently than wild type cells to a stimulus such as 1% FCS (Figure 34a).
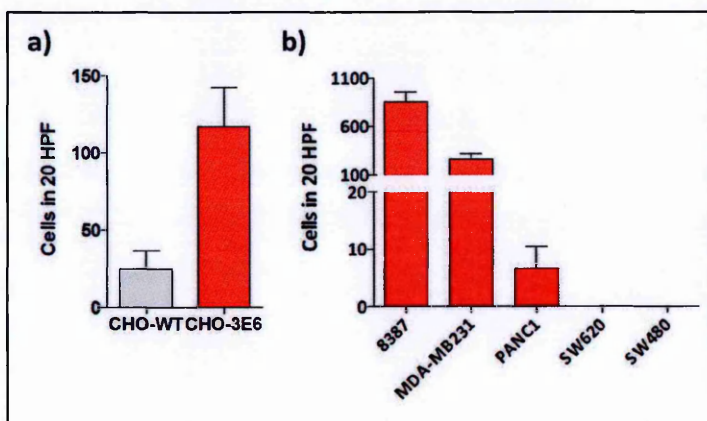
Figure 34 Motility of CHO and human cancer cell lines. Migratory response of cells to 1% FCS. a) migration of CHO-WT and CHO-3E6 cells; b) migration of human cancer cell lines.

In addition, we tested some of the cancer cell lines expressing different levels of MSF, as evaluated by RT-qPCR (Figure 30). As shown in Figure 34b, some of the cancer cell lines tested migrate to 1% FCS. Interestingly, a significant correlation was found between the levels of MSF expression evaluated by RT-qPCR and the migratory response (Pearson r=0.9462; p=0.015) (Figure 35).
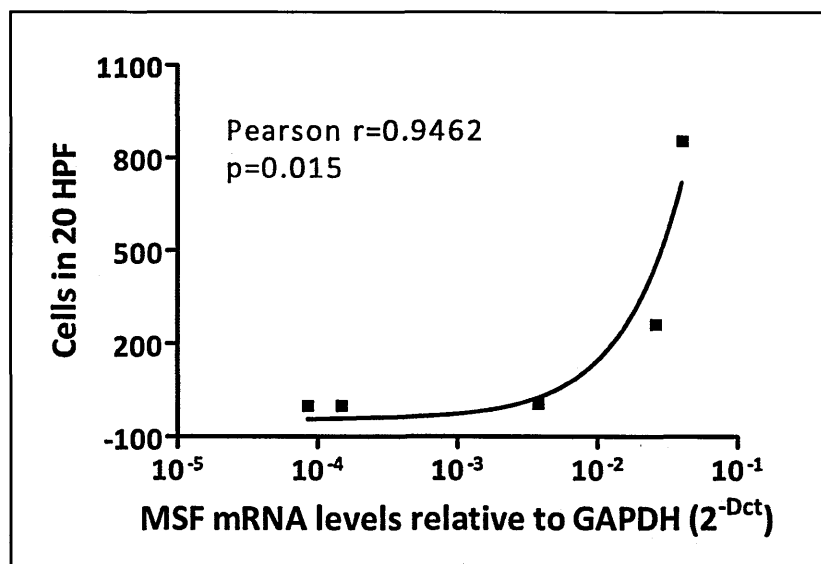

Figure 35. Correlation between MSF mRNA expression and migratory response of tumor cells. Migratory response of cells to 1% FCS was correlated to the expression of MSF mRNA. A significant correlation (Pearson r=0.9462, P=0.015) was observed.

4.2.3 *In vivo* transplantation of MSF transfected tumour cells

To investigate a possible cancer promoting role of MSF we took advantage of the transfected CHO cells expressing MSF (CHO-cl1 and CHO-3E6). Given our observation on MSF expression in M2-like pro-tumoral macrophages, we expected that MSF expression could eventually increase macrophage recruitment in the tumour. Thus we first investigated whether human MSF could exert its properties on murine macrophages. To this aim we performed a chemotaxis experiment with murine peritoneal macrophages. As shown in Figure 36, murine peritoneal macrophages can significantly migrate in response to both Lot 4 and Lot 5 of human MSF.
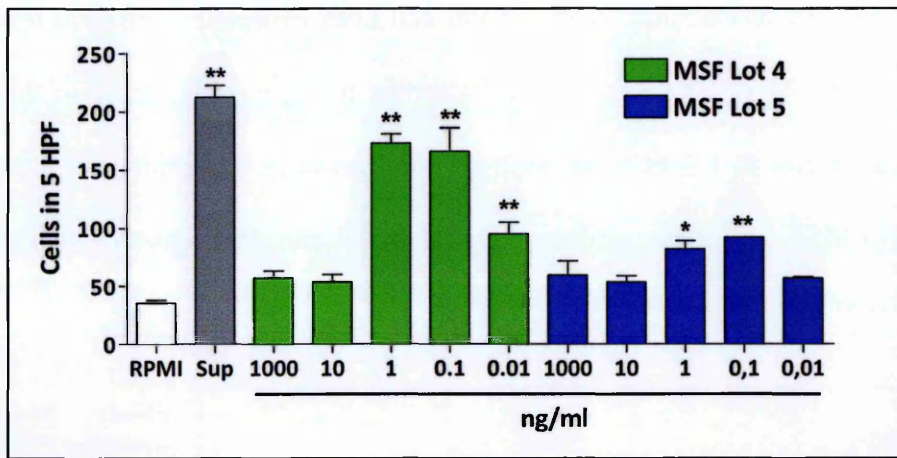
Figure 36. Migration of mouse peritoneal macrophages to human MSF. Macrophages were plated in the upper compartment of a microchamber. Lower wells contained RPMI w/o FCS as negative control and supernatant from CHO 3E6 cell (sup) as positive control. Different concentration of purified MSF from 1000 to 0.01 ng/ml (in green Lot 4 and in blue Lot 5) were tested. After 4 h incubation at 37°C cells in 5 HPF were counted.. * $p<0.05$; ** $p<0.01$, one way Anova.

CHO-cl1 and CHO wild type cells were then injected in SCID mice, and the *in-vivo* growth of tumours followed over time. In a first experiment mice were injected with $3x10^6$ cells from each cell line. In that case the tumours appeared very rapidly (latency time approximately 8-10 days) in all the animals without appreciable differences in tumour takes. Despite this, we found a significant difference ($p<0.05$ Student's t test) in tumour volumes between CHO-WT and CHO-cl1 at day 16, 19 and 21 after transplantation (Figure 37a). The experiment was then conducted with $5x10^5$ and $5x10^4$ cells: no significant differences in latency and tumour growth were observed when mice were treated with $5x10^5$ cells (Figure 37b), but there was a trend toward a higher growth rate for CHO-cl1 cells.

When mice were treated with $5x10^4$ cells, a significant difference ($p<0.05$) in tumour growth between CHO-cl1 and CHO-WT cells was observed at day 21, 24 and 36 after transplantation (Figure 38), while no differences in latency were observed. Given these results we treated mice with CHO-3E6, that can produce 5 times more MSF compared to CHO-cl1. Mice were treated intramuscularly with $5x10^4$ CHO-3E6 cells and CHO-WT cells. A significant difference (***$p<0.001$) in tumour growth between the two groups was observed at day 16, 18, 21, 24 (Figure 39). Similarly, when mice were treated with $5x10^3$,

a tendency for higher growth rate of tumours derived from MSF-expressing cells was observed but difference was statistically significant only at day 31 (not shown).

The expression of MSF in tumours derived from CHO-WT or CHO-3E6 cells was evaluated by PCR. Tumours were excised from the animals at day 24 and results of the PCR analysis are reported in Figure 40. cDNA, retrotranscribed from total RNA extracted from tumours derived from CHO-WT (lanes 1-8) or CHO-3E6 (lanes 9-16) cells, was amplified with primers for human MSF and for hamster FN1. As shown in Figure 40, tumours derived from CHO-3E6 cells express human MSF even after 24 days of growth in mice. All the tumours express, as expected, hamster FN1.
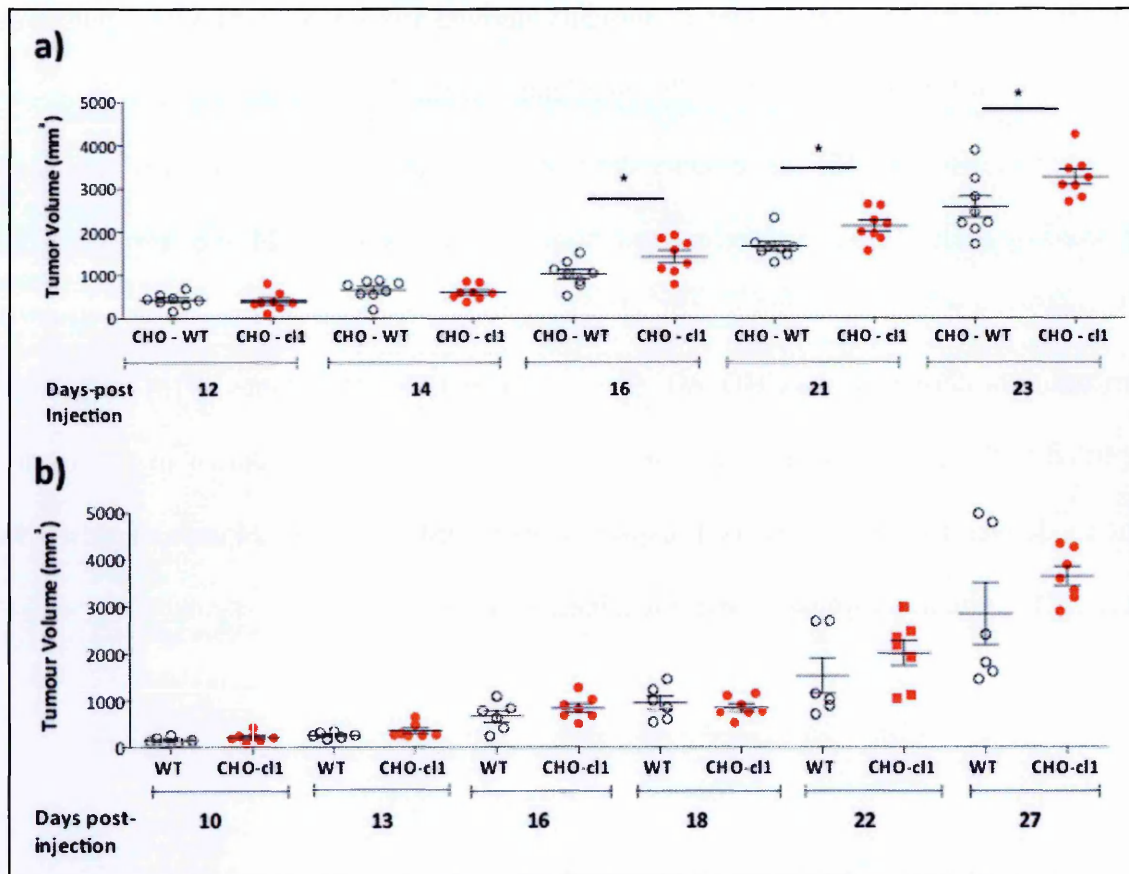
Figure 37. *In vivo* **growth of CHO-cl1 and CHO-WT cells.** CHO-cl1 (n=7) and CHO-WT (n=7) were injected intramuscularly in CB17/SCID. Two different doses were used $3x10^6$ (panel a) and $5x10^5$ (panel b). * p<0.05 (Student's *t* test);
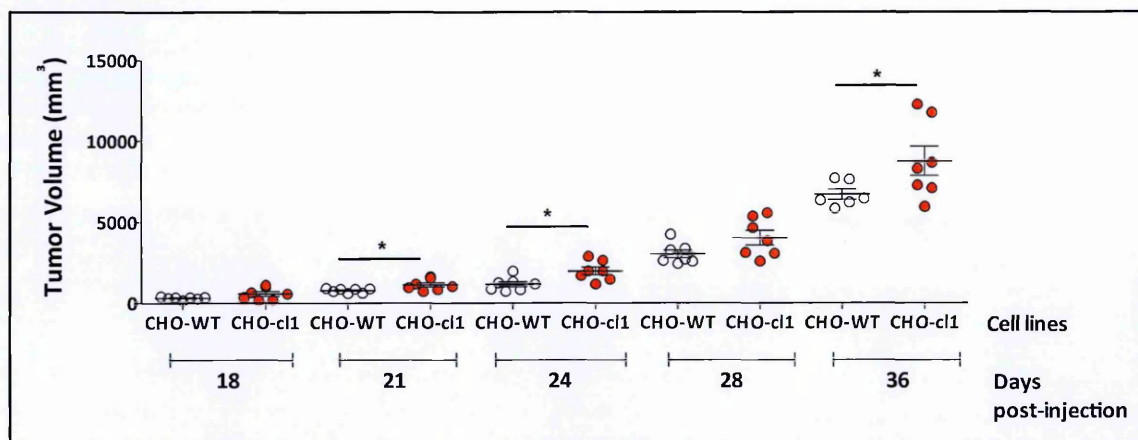
Figure 38. In vivo growth of CHO-cl1 and CHO-WT cells. CB17/SCID Mice were injected intramuscularly with $5x10^4$ CHO-WT (empty circle, n=7) or CHO-cl1 (red circle, n=7) cells. Tumour volume was measured at the indicated time points. Volumes measured for each animal are reported, black line represent the mean volume ±SEM for each group and time point. (*p<0,05 Student's *t* test).
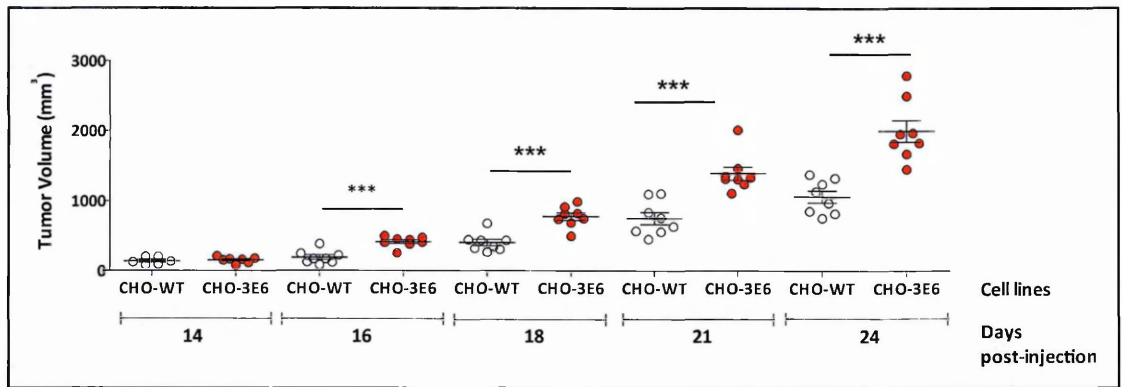
Figure 39. *In vivo* **growth of CHO-3E6 and CHO-WT cells.** Mice were injected intramuscularly with $5x10^4$ CHO-WT (empty circle, n=8) and CHO-3E6 (red circle, n=8) cells. Tumour volume was measured at the indicated time points and volumes measured for each animal are reported. Black line represents the mean volume ±SEM for each group and time point. (***p<0,001).
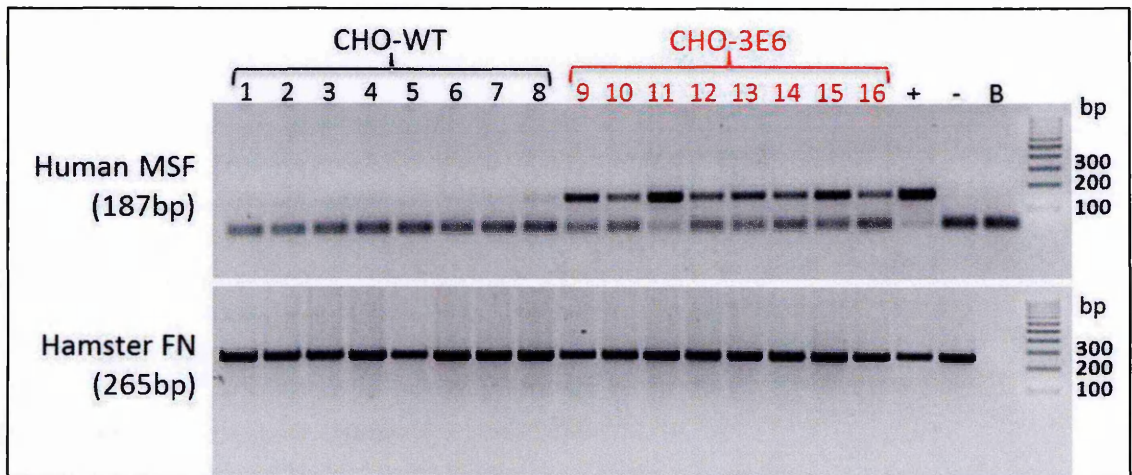
Figure 40. MSF expression in tumours derived from CHO-3E6 and CHO-WT cells. Tumours from different animals (each number represent a mice) were excised at day 24 after intramuscular injection with $5x10^4$ CHO-WT or CHO-3E6 cells. cDNA obtained by retrotranscription of total RNA was amplified analysed by agarose gel electrophoresis to evaluate expression of human MSF and hamster Fibronectin.

4.3 MSF expression in mouse and human tumours

Given the selective expression of MSF by M2 macrophages and M2-like TAMs we were interested to investigate MSF expression in human tumours. We thus took advantage of the polyclonal and monoclonal antibodies developed in this thesis to investigate MSF expression in tumour tissues.

4.3.1 Immunohistochemical analysis of MSF expression in mouse model

Initially we analysed MSF expression in sections from the tumours derived from CHO-WT and CHO-3E6 cells transplanted *in vivo*. Two antibodies were used, namely RAB2.3 and MoAb_E10. Tumours were analysed at day 24 after injection. A positive signal was observed after staining with RAB2.3 of tumor sections derived from CHO-3E6, while sections from CHO-WT tumors were substantially negative (Figure 41a). A similar scattered staining was observed when the monoclonal antibody MoAb_E10 was used (Figure 41b). Different mechanisms could explain the higher growth rate of tumours derived from MSF-expressing cells compared to CHO-WT. It is known and we confirmed here that MSF has a motogenic activity for different cells (Schor & Schor, 2010) and in particular we showed that MSF can attract macrophages (e.g. Figure 36). Thus we analysed the expression of F4/80, a well-known macrophage marker, in tumours derived from CHO-WT and CHO-3E6 cells. However no differences were observed in terms of percentage of F4/80$^+$ cells evaluated by FACS (not shown), nor in term of F4/80 positive area evaluated by immunohistochemistry (Figure 42). Since MSF has also been described as pro-angiogenic factor (Schor et al, 2001), it would be interesting to analyse the expression of CD31, a marker of endothelial cells, in tissue samples from tumours derived by injection of CHO-WT and CHO-3E6 cells. However preliminary results on three tumours did not show any significant difference in the levels of CD31 positive cells (data not shown), and further experiments will be necessary to address this point.
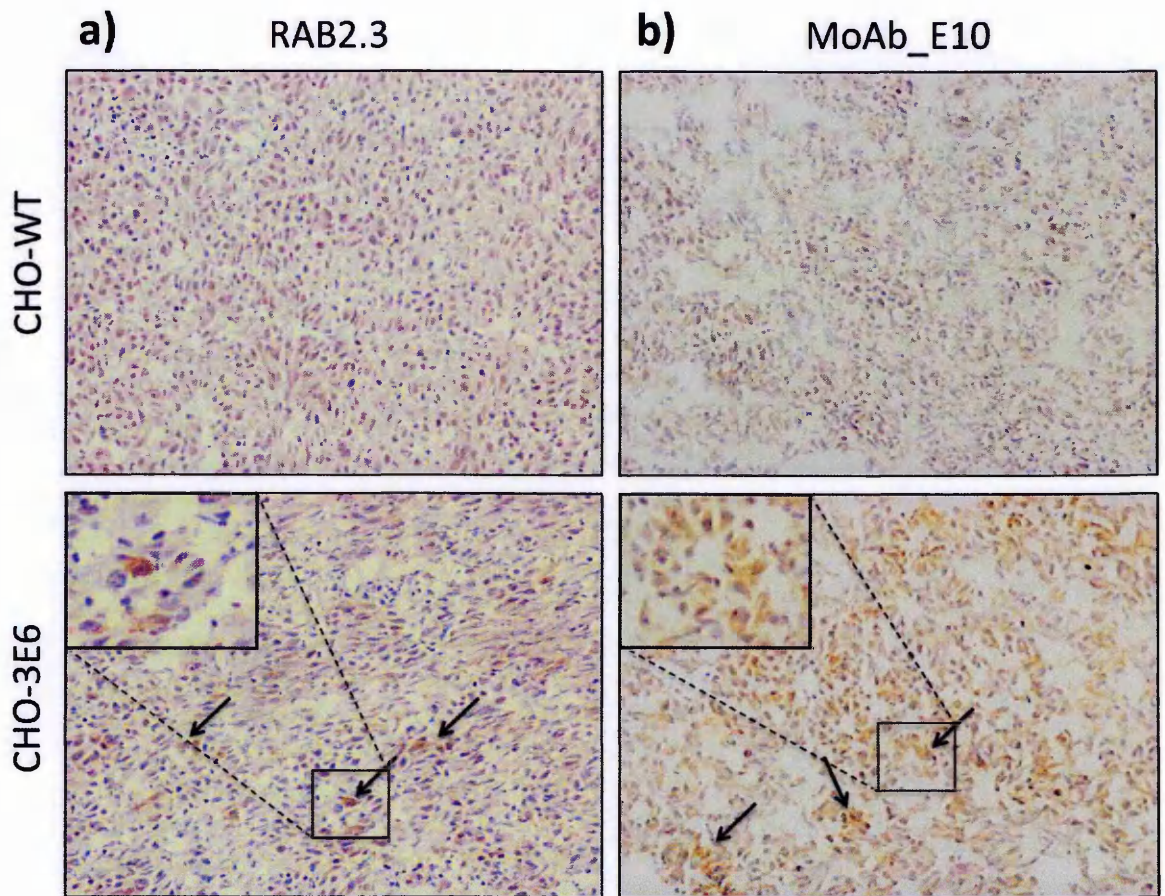
Figure 41. Immunohistochemical analysis of MSF expression in tumours derived from CHO-WT and CHO-3E6 cells. Tumours were excised 24 days after injection of CHO-WT and CHO-3E6 cells in mice. Tumour sections (10x) were stained with a) RAB2.3 (5 µg/ml) or b) MoAb_E10 (1,5 µg/ml). DAB was used to develop the staining. Arrows indicate some MSF expressing cells (brown); inserts are higher magnifications of selected areas.
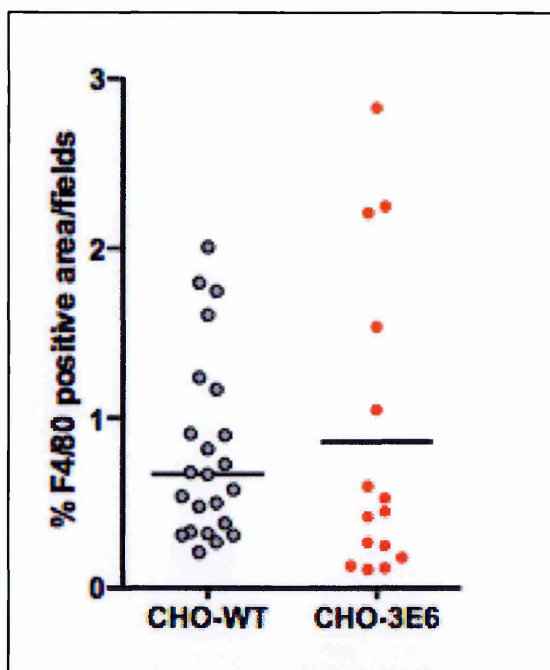
Figure 42. Percentage of F4/80 positive cells. Analysis of F4/80 positive area /fields on sections from tumours derived from CHO-WT and CHO-3E6 cells. Black lines represent median values of the two distributions.

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4.3.1 Immunohistochemical analysis of MSF expression in human tumours

We then proceeded to analyze MSF expression in formalin-fixed and paraffin-embedded human primary lung, breast and colorectal cancers with MoAb_E10. Tumoral as well as stromal cells were immunoreactive in all analysed tissues. In tumoral cells MSF is heterogeneously expressed. A preliminary evaluation performed by an expert histologist indicate that endothelial cells lining blood vessels, cancer-associated fibroblasts (CAFs), and a subset of immune cells of the innate system also express MSF (Figure 43). These observations need to be confirmed by further experiments identifying with certainty the cells expressing MSF in tissue sections. Interestingly, in both lung and breast cancer CD68+ infiltrating macrophages were also immunoreactive for MSF. In the adjacent alveolar tissue, macrophages were found immunoreactive for both CD68 and MSF (Figure 44)

MoAb_E10

Breast
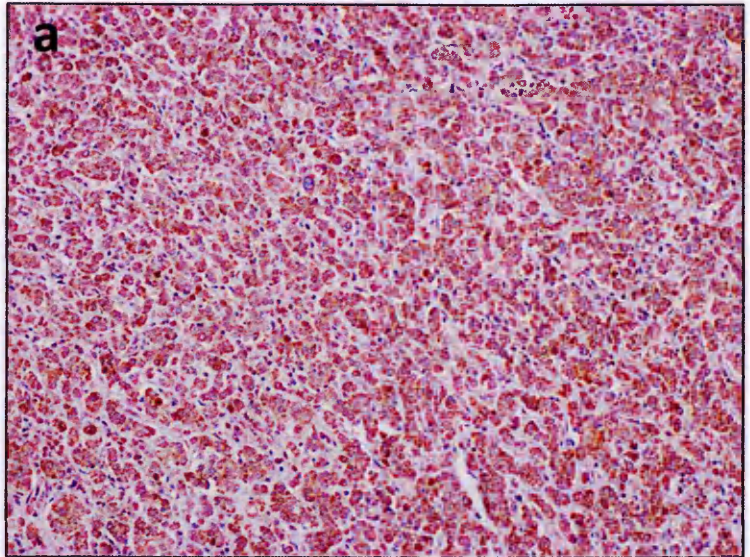
Colon
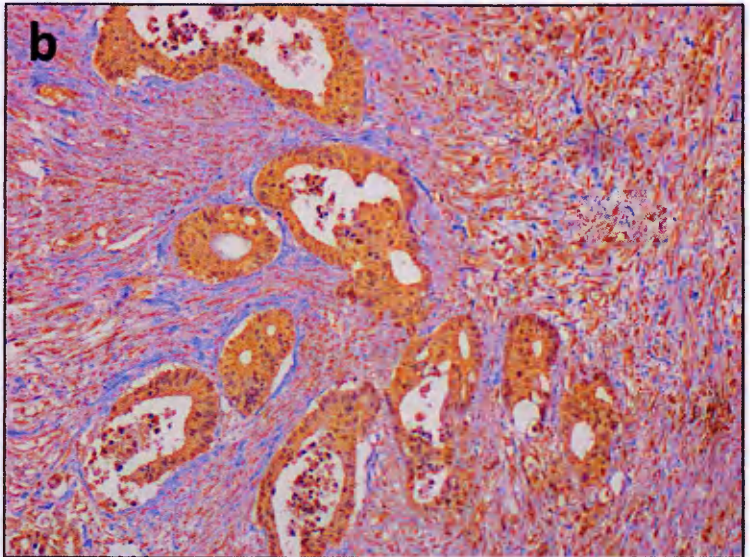
Lung
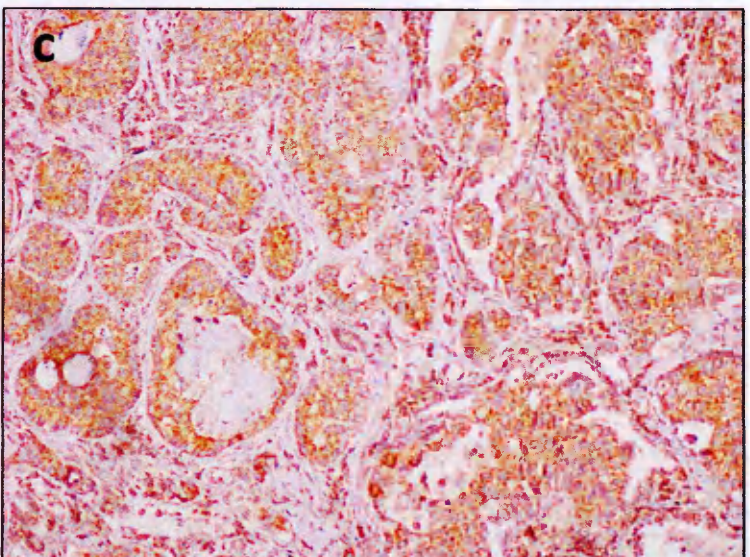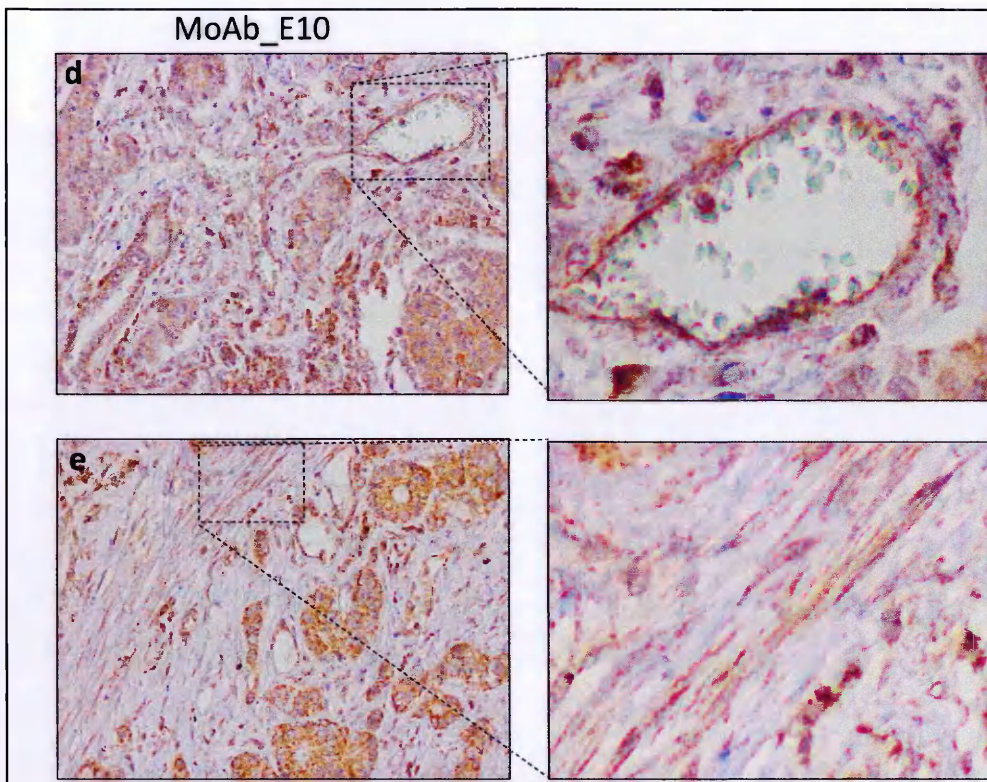
Figure 43. Immunohistochemical analysis of MSF expression in tumours derived from human breast ductal adenocarcinoma, colon carcinoma and non-small cells lung cancer. Tumour sections were stained with MoAb_E10 (2.5 µg/ml). a) breast ductal adenocarcinoma (10x); b) colon carcinoma (10x); c) non-small cells lung cancer (10x). d) & e) are 20x images of non-small cells lung cancer: endothelial and stromal component (d), and stromal and immune cells (e).
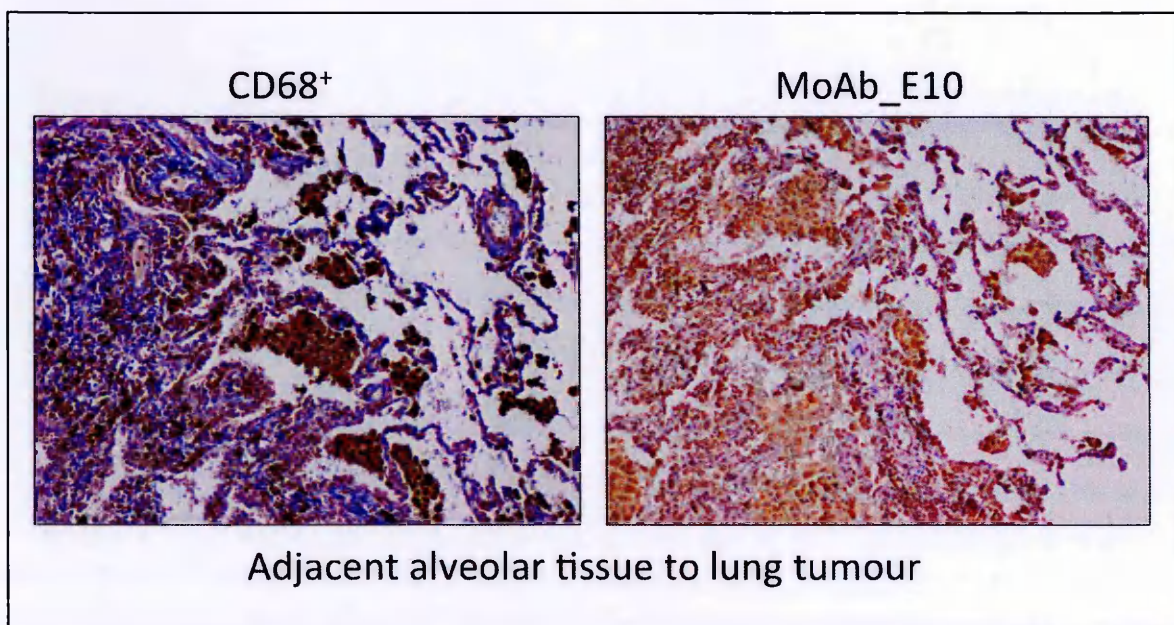
Figure 44. Immunohistochemical analysis of MSF expression in alveolar tissue adjacent to tumour area in lung cancer. Tumour sections were stained with anti-CD68 (780 µg/ml) and MoAb_E10 (2.5 µg/ml). Alveolar tissue adjacent to tumour area shows alveolar macrophages ($CD68^+$) that express MSF protein.

4.3.2 *In situ* hybridization on tumour tissues

To confirm the wide staining with MoAb_E10 observed in different tumour specimens, we performed *in situ* hybridization (ISH). CHO-3E6 cells and CHO-A3 were transplanted into recipient mice and tumours were excised 24 days after injection. Tissue sections of tumours derived from both CHO-A3 and CHO-3E6 cells were prepared and processed for ISH. As shown in Figure 45, a clear signal was detectable in sections derived from CHO-3E6 hybridized with MSF antisense probe, whereas no signal was observed with MSF sense probe on all tissue sections, irrespective of whether they were derived from CHO-WT or CHO-3E6 tumours. These results indicate that ISH represents an useful assay to evaluate MSF expression in tumour tissues. Therefore, we performed ISH on human breast cancer samples obtained from the Pathological Anatomy Department, Humanitas Clinical and Research Center, Milan, Italy. In these samples derived from human histological sections we found high levels of MSF expression in transformed cells (Figure 46), confirming the high expression of MSF observed by immunohistochemistry.
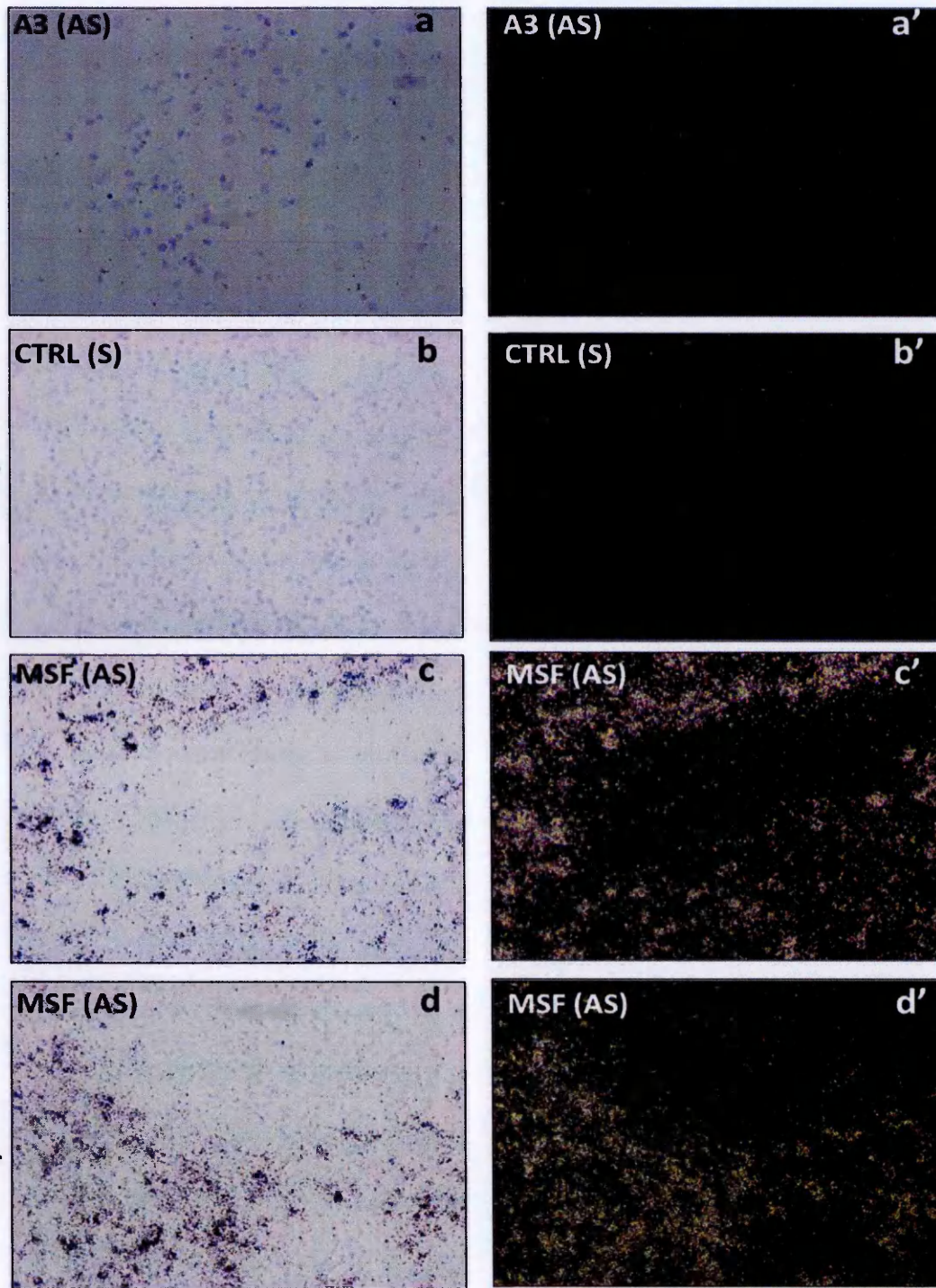
Figure 45. Analysis of MSF mRNA expression by *in situ* hybridization. Analysis of MSF mRNA expression in tissue sections derived from CHO-3E6 cells or control cells (CHO-A3). Panel a and a' are respectively the haematoxylin staining and ISH with the antisense riboprobe (AS) on sections from the control tumours; b and b' are haematoxylin staining and ISH with the sense riboprobe (S) on sections from CHO-3E6 tumours; c and c' and d and d' are haematoxylin staining and ISH with the antisense riboprobe on sections from CHO-3E6 tumours. Original magnification 20x.
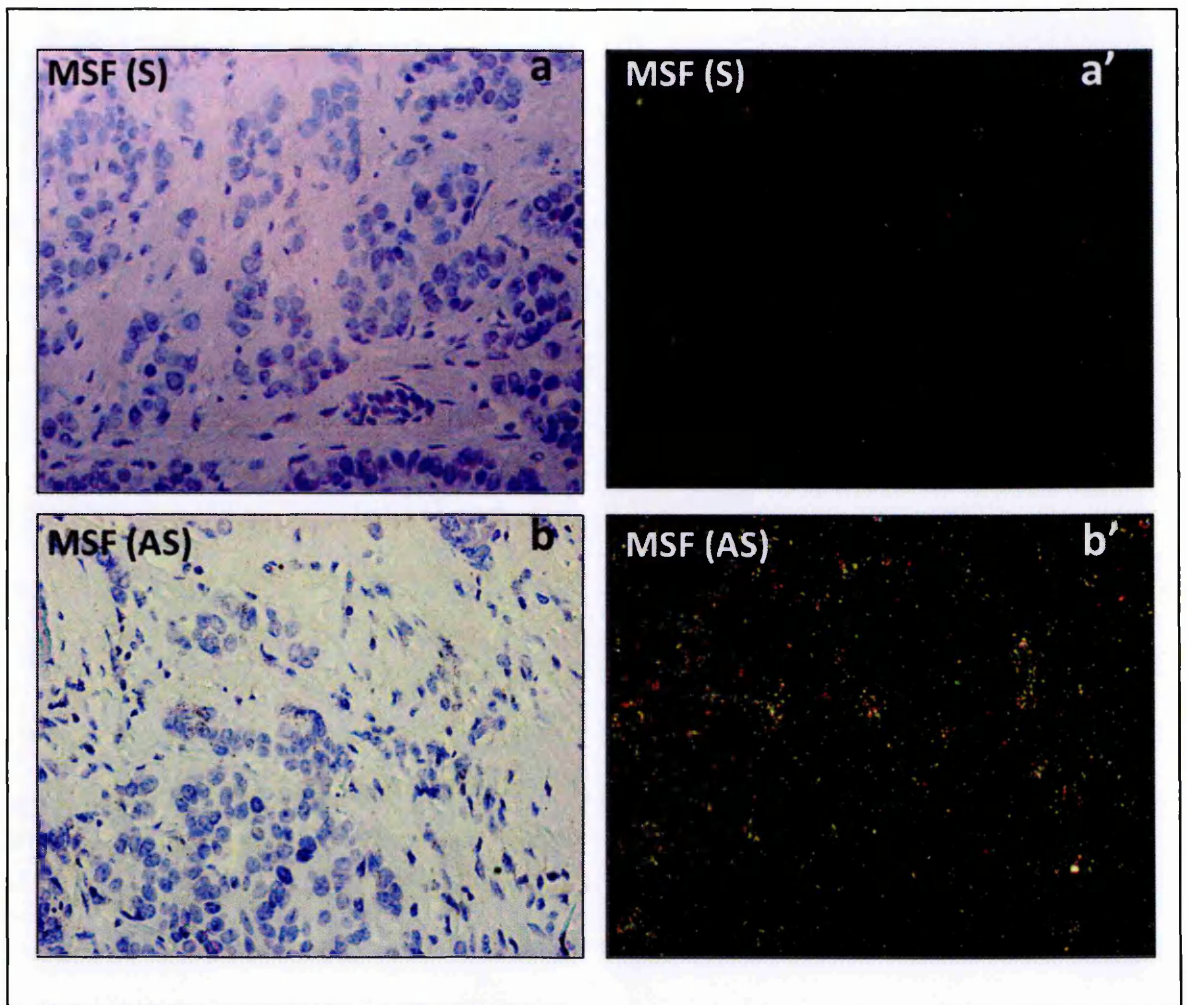
Figure 46. Analysis of MSF mRNA expression by *in situ* **hybridization in human breast cancer.** Immunohistochemical analysis (a, b) and *in situ* hybridization (a', b') of human breast cancer: in panel a and a' is shown the negative control using the sense riboprobe (S); in panels b and b' is shown MSF mRNA in tumour tissue using the antisense riboprobe (AS).

5 DISCUSSION

The aim of this work was to provide new insights on migration stimulating factor, a molecule produced by tumour-associated macrophages. Here we show the generation of new original reagents (recombinant protein, polyclonal and monoclonal antibodies) to study human recombinant MSF, a first characterization of the biological properties of the recombinant protein and the analysis of MSF expression in human tumours.

Much work has been done to characterize the role of TAMs in tumour biology, and a general consensus has now been reached in considering TAMs as strong supporters of tumour growth (Steidl et al, 2010). Recently Solinas *et al.* demonstrated that monocytes exposed to tumour-conditioned medium can acquire M2 polarization. In agreement, gene profiling data indicated that TAMs have an M2-like polarization. Taken together these observations suggest that the tumour itself can condition infiltrating macrophages to acquire a pro-tumoral M2-like phenotype. Among the genes selectively associated to M2 polarization of macrophages is migration stimulating factor (Solinas et al, 2010).

MSF is an oncofetal isoform of human Fibronectin 1 originally described as expressed by fibroblasts, keratinocytes and vascular endothelial cells during the foetal life but not by healthy adult cells (Schor & Schor, 2010). The expression observed by us and by others (Deng et al, 2013; Schor & Schor, 2010) in tumor cells suggests that MSF could be part of the transcriptional program associated with neoplastic trasformation. This is also supported by the observation that M2-polarized pro-inflammatory and pro-tumoral macrophages can produce MSF, while M1 macrophages, that exert a strong tumoricidal activity, do not express this molecule. Accordingly with the theory that associates inflammation and cancer, it would be worthwhile to investigate whether MSF expression is up-regulated also in chronic inflammatory diseases from which cancer progression may occur.

The observation, made by gene profiling and confirmed in this study, of a selective expression of MSF by M2-like tumour associated macrophages accounts for our interest in

this protein as potential marker for M2-like polarization. Since no commercial reagents were available to study MSF, considerable efforts were aimed at expressing and purifying recombinant human MSF and producing polyclonal and monoclonal antibodies recognizing this protein. Given the almost complete identity of MSF and FN1 and the high conservation of FN1 between humans and mice, the generation of antibodies was an important challenge and was made possible by outsourcing part of the activity. We obtained both polyclonal and monoclonal antibodies recognizing with specificity and a good sensitivity human MSF and not cross-reacting with FN1.

A second important issue was the production and purification of human MSF. Also in this case we took advantage of external partners that cloned the complete cDNA sequence of human MSF and helped in the setting up of the purification process. In addition we outsourced the production of His-MSF, a polypeptide comprising a hundred amino acids in the COOH terminal portion of MSF, including the specific decapeptide used as that is unique to MSF.

To evaluate the functional activity of purified MSF, we initially focused on its motogenic properties. The recombinant MSF produced and purified during this project has a motogenic activity for Panc1 cells used as a model system. Panc1 cells migrate to MSF as much as to a standard motogenic stimulus represented by foetal calf serum. We observed an heterogeneity in the migratory response of tumour cells, with the 8387 fibrosarcoma and a breast cancer cell line characterized by the highest motility, while, on the contrary cell lines derived from colon cancer have a modest or no migratory response. The same set of tumour cell lines was analysed by RT-qPCR to quantify MSF expression. Interestingly, a significant correlation was observed between the levels of MSF mRNA expression and the migratory capacity of the cell types analysed, suggesting that MSF could act as an endogenous motility factor. This observation may have an important implication for the process of metastasis formation, in fact tumor cell motility plays a vital role in the process of tumor invasion and metastasis. In order to invade and metastasize,

tumor cells must actively migrate out of the primary tumor and cross the extracellular matrix. Several substances, produced either by tumor or stromal cells, can stimulate cell locomotion, including for example members of the FGF family or cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF). A recent proteomic analysis of colorectal cancer (CRC) and non-tumorigenic tissues reveals a differential protein signature according to disease (CRC vs non-tumorigenic tissues) and stage (early vs late stage) (Sethi et al, 2015). Among the differentially expressed proteins, fibronectin showed the highest expression levels in CRC samples. Fibronectin is known to be involved in cell adhesion and migration (Pankov & Yamada, 2002), providing a pathway for stromal cell migration during embryogenesis, morphogenesis and wound healing. Similarly, fibronectin expression and deposition at sites of cancer can provide a path to tumor cells to move on. In this context it is tempting to speculate that MSF could exert a similar function, providing a road to tumor cells, thus favouring invasion and metastatization. This hypothesis needs to be confirmed by further experiments involving for instance the silencing of MSF in cancer cells.

The induction of a foetal-like migratory phenotype in adult fibroblasts is one of the properties of MSF that could be associated with neoplastic transformation (Schor & Schor, 1987), thus it might be interesting to study the direct involvement of MSF in the induction of epithelial-mesenchymal transition. To this aim we are generating transfectants from Panc 1 cells, which express very low levels of endogenous MSF, to have cells lines with different capability to produce MSF. Once injected orthotopically in mouse pancreas, we could evaluate the incidence, growth rate and invasiveness of pancreatic cancer cell lines producing different levels of MSF. The monoclonal antibodies generated in this Thesis project were not tested jet as blocking antibody, but in the case one of them has this property it could be used to demonstrate the involvement of MSF in tumour progression.

The factors that could influence the heterogeneity in MSF mRNA expression by different cancer cells are not known. It is known that M-CSF produced by tumour cells is

responsible for MSF expression in macrophages exposed *in vitro* to tumour cell conditioned medium (Solinas et al, 2010). In addition cytokines promoting M2 polarization can regulate MSF expression in macrophages. TGF-β, a pro-tumoral cytokine mediating tumour-stroma interactions, has been described to induce MSF expression in fibroblasts from cancer patients or healthy subjects (Schor & Schor, 2010). We did not analyse the production of these mediators in our setting and in general further investigation on the regulation of MSF expression in tumour cells would likely be helpful to understand the relationship between MSF itself and cancer.

Beside tumour cells, we observed a significant migratory response of peripheral blood monocytes to recombinant MSF. Similarly to chemokines, MSF presents a characteristic bell-shape response curve and exerts its effect in the nanomolar range of concentration. An exhaustive analysis of target cells, i.e. lymphocytes, neutrophils, dendritic cells, is still missing and will be objective of further investigations. It has not been established jet whether MSF stimulates directional (chemotactic) and/or random (chemokinetic) motility of monocytes and tumor cells and no indications on a putative MSF receptor are available.

Several different integrins are described as putative receptor for fibronectin, such as α4β1, interacting with the IIICS region, and α5β1, interacting with the central region of FN1. However the domains involved in the interaction of FN1 with integrins are not conserved in MSF. On the other side MSF maintains in its sequence some IGD motifs that are described to be motogenic (Schor et al, 1999) and to interact with αvβ3 integrin present in the focal adhesion (Millard et al, 2007). This interaction involves the tyrosine phosphorylation of focal adhesion kinase (ppFAK125) inducing an arrangement of the actin filaments in the cytoskeleton and consequently the migration. The RGD motifs present in fibronectin and not conserved in MSF also interact with αvβ3 integrin, but it is noteworthy to underline that interaction of RGD motifs with αvβ3 integrin results in loss and not in gain of motogenic activity, likely because of the inherent rigidity of these domains.

The bell-shaped migratory response observed in our set of experiments suggest a possible analogy with chemokines, thus interaction of MSF with chemokine receptors also deserves further investigation using for instance transfected cells expressing a specific chemokine receptor or by means of blocking antibodies against different chemokine receptors.

We also found that human MSF can act on the murine system, stimulating migration of murine peritoneal macrophages. The optimal activity has been obtained with 1-0.1 ng/ml, a slightly higher concentration, but essentially comparable to what observed in human monocytes. A murine MSF has been identified (P. Brennecke et al, personal communication), characterized by the presence of a hypothetical dodecapeptide, instead of a decapeptide, at the C-terminus. The homology between the protein sequences of human MSF and the hypothetical murine counterpart is approximately 92,7%. The few mismatches between the two molecules are located in the motogenic domain (Gel-BD) of the protein. This could be likely responsible for the different efficiency in terms of motogenic activity of human MSF on human or mouse macrophages. This observation suggests that, even if with several limitations, the murine model can be used to test the biological role of MSF in tumours.

To evaluate a pro-tumoral role of MSF we investigated the in vivo growth rates of tumours derived from cells expressing or not human MSF. Injection of transfected cells expressing MSF into CB 17/SCID mice led to the development of tumours with higher growth rate compared to the control counterparts. It has been described that MSF does not exert mitogenic effects on tumour cells (Deng et al, 2013), and we confirm this observation on our MSF-expressing CHO cells and CHO-WT cells. Thus, given the migratory effect of MSF on monocytes, an obvious mechanism to explain the higher growth rate observed in vivo was the possible attraction and accumulation of TAMs. However, when tumour sections were analysed by immunohistochemistry, we did not observe any difference in the percentage of macrophages infiltrating the experimental tumours. In the same setting the

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evaluation of angiogenesis did not evidenced any significant difference between tumours expressing or not MSF. This latter observation need further investigation since a proangiogenic role is described for MSF. We could not exclude the release by stromal cells of other angiogenic factors that could overcome the presence or not of MSF. Subcutaneous injection of MSF expressing cells in a matrigel will probably allow better to define the pro-angiogenic role of MSF protein.

The observations in this setting indicate that macrophage infiltration is not sufficient to explain the higher tumour growth rate observed *in vivo*. Even if we did not observe any difference in the *in vitro* growth rate of cells expressing or not MSF, we could not exclude a direct effect of MSF *in vivo* on tumour or stromal cells, for istance promoting the release of growth factors. In addition a skewing of M1 to M2 polarized macrophages could eventually determine the differences observed in the growth rate. Thus the mechanisms involved in the growth differences observed *in vivo* need further investigations.

The observation that MSF was expressed by different tumour cell lines as well as by M2-like TAMs, prompted us to analyse MSF expression in histological sections derived from human cancers. We found an extensive staining with MoAb_E10, recognizing human MSF, in breast, lung and colon cancers, regarding tumour, stromal and endothelial cells. *In situ* hybridization confirmed the high level of MSF expression in tumour sections. The immunohistochemical analysis also confirmed the positivity of a proportion of infiltrating macrophages identified by the expression of CD68. These observations indicate that the anti MSF antibodies generated here can be used to identify sub-population of TAMs, likely M2-like TAMs, and to follow the changes over the time for this population. As a matter of fact pre-clinical studies demonstrate that re-education of TAMs can endow them with the capacity to kill tumour cells (Duluc et al, 2009; Mantovani et al, 2014), thus more often TAMs are target for therapeutic approaches aimed to their re-education (Getts et al, 2014).

While an extensive staining for MSF was observed in lung and breast tumour specimens, a more heterogeneous immunostaining was seen in colon cancer sections, in

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agreement with the lower MSF expression observed in colon cancer cell lines. In this regards, infiltrating macrophages in colon cancer represent an exception in the field of tumour-associated macrophages. In fact contrasting results are reported, with some observations reporting the association of TAMs with a poor prognosis, while in other cases TAMs infiltrating colon cancers have a protective role (reviewed in (Erreni et al, 2011; Mantovani & Allavena, 2015). This suggests again the importance of tumour microenvironment in determining the polarization of macrophages and the effects this may have on the production of soluble mediators such as MSF.

The presence of MSF in the serum of breast cancer patients has been demonstrated previously using a bioassay for the measurement of migration stimulating activity for fibroblasts (Picardo et al, 1991). Apart from this bioassay, no systems for the measurement of MSF levels in biological fluids are available. Therefore, we set up a sandwich ELISA using a combination of anti FN1 and rabbit polyclonal anti-MSF antibodies. With this system we were able to measure MSF in plasma better that in serum of healthy volunteers. This observation potentially represent an important proof-of-principle of the possibility to measure MSF in biological fluids. Ongoing efforts are directed to investigate whether MSF could be a marker of neoplastic transformation in humans.

TAMs exert several pro-tumoral functions, promoting tumour proliferation, angiogenesis, immune suppression, tissue remodelling and metastasis, and eventually also resistance to therapy (Biswas & Mantovani, 2010; Mantovani et al, 2014; Pollard, 2004). MSF is produced by M2-like TAM and is upregulated both in cancer and stromal cells, thus is part of the tumoral microenvironment. In addition, expression by M2-like pro-tumoral TAMs suggests that MSF could represent an attractive marker of M2 polarization. Given that TAMs are promising target for anticancer therapy, the development of an antibody that can identify M2 polarized cells would be of considerable interest.

In the era of personalized medicine the tailoring of medical treatments to the individual characteristics is acquiring more and more relevance in the set-up of new

therapeutic strategies. In this context the possibility to identify M2 polarized macrophages infiltrating a tumor may have a particular relevance. To this aim it would be interesting to perform an extensive immunohystochemical analysis on human tumors with different origin in order to evaluate the levels of M2 polarized, MSF-expressing tumor associated macrophages. This could allow to obtain a "molecular profile" for each patient by combining the levels of MSF in biological fluids, evaluated with a sandwich ELISA to be developed, and the immunohistochemical evaluation of M2-polarized TAMs. Thus MSF could have a significant diagnostic e prognostic value. MSF levels could also be analysed in relation to tumour staging or on the basis of the chemotherapeutic treatment of patients, in order to correlate MSF expression with tumour progression. On the basis of these analysis we expect to define whether MSF could represent a useful marker for tumour progression.

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Acknowledgements